Influence of Dietary Fat Content on Vascular Reactivity and Expression Profiles: Novel Role of Vascular Cyp2E1

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List of Abbreviations

Actb	Actin beta gene
ADP	Adenosine diphosphate
α_1	Alpha$_1$
ANOVA	Analysis of variance
AngII	Angiotensin II
AA	Arachidonic acid
AU	Arbitrary units
bp	Base pair
BH_4	(6*R*) 5, 6, 7, 8-tetrahydro-L-biopterine
BMI	Body Mass Index
BK_{ca}	Ca^{2+}-activated K^+ channels
Ca^{2+}	Calcium
Car3 and Car3	Carbonic anhydrase 3 gene and protein
Cidec and Cidec	Cell-death-inducing DFFA-like effector c gene and protein
CO	Carbonic monoxide
CD	Control diet
CDS	Coding sequence
CRP	C-reactive protein
cAMP	Cyclic adenosine 3`, 5`-monophosphate
cGMP	Cyclic guanosine 3`, 5`-monophosphate
COX	Cyclooxygenase
$Cyp2E1^{-/-}$	Cyp2E1-deficient
Cyp2E1 and Cyp2E1	Cytochrome P450, family 2, subfamily E, polypeptide 1 gene and protein
DNA	Deoxyribonucleic acid
DAG	Diacylglycerol
Dgkg and Dgkg	Diacylglycerol kinase, gamma gene and protein gene and protein
DIO	Diet-induced obesity
DETC	Dietyldithiocarbamate
Dpp10 and Dpp10	Dipeptidylpeptidase 10 gene and protein
ER	Endoplasmic reticulum
EC	Endothelial cell
eNOS or NOS III	Endothelial nitric oxide synthase
EDRF	Endothelium-dependent relaxing factor
EDCF	Endothelium-derived contracting factor
EET	Epoxyeicosatetraenoic acid
Fe^{3+}	Ferric iron
Fe^{2+}	Ferrous iron
Fkbp5 and Fkbp5	FK506 binding protein 5 gene and protein

Fmo3 and Fmo3	Flavin containing monooxygenase 3 gene and protein
GO	Gene Ontology
GTT	Glucose tolerance test
GTP	Guanosine-5`-triphosphate
HDL	High density lipoprotein
HFD	High fat diet
HFD/CD	High-fat diet/Control diet
H_2O_2	Hydrogen peroxide
HETE	Hydroxyeicosatetraenoic acid
•OH	Hydroxyl radical
iNOS or NOS II	Inducible nitric oxide synthase
IP_3	Inositol 1, 4, 5-triphosphate
IL-1	Interleukin-1
IL-6	Interleukin-6
K^+	Potassium
kDa	Kilo Dalton
L-Arg	L-arginine
L-NAME	NO-synthase inhibitor N^G-nitro-L-arginine methylester
Lepr and Lepr	Leptin receptor gene and protein gene and protein
Lep^{ob}/Lep^{ob}	Spontaneous mutation in the *leptin* gene
LOX	Lipooxygenase
LDL	Low density lipoprotein
Lyve1 and Lyve1	Lymphatic vessel endothelial hyaluronan receptor 1 gene and protein
mRNA	Messenger ribonucleic acid
NAPQI	*N*-acetyl-*p*-benzoquinone
Nrg1 and Nrg1	Neuregulin 1 gene and protein
nNOS or NOS I	Neuronal nitric oxide synthase
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NEFA	Nonesterified fatty acid
O_2	Oxygen
$ONOO^-$	Peroxynitrite
PAI-1	Plasminogen activation inhibitor-1
PBS	Phosphate buffered saline
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PLA_2	Phospholipase 2
PLC	Phospholipase C
PL	Phosholipid
Poly(A) tail	Polyadenylation tail
pre-mRNA	Precursor messenger ribonucleic acid

PGI_2	Prostacyclin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PKC	Protein kinase C
PKG	Protein kinase G
P2rx1 and P2rx1	Purigenic receptor P2X. ligand-gated ion channel gene and protein
Pdk4 and Pdk4	Pyruvate dehydrogenase kinase, isoenzyme 4 gene and protein
qRT-PCR	Quantitative real-time polymerase chain reaction
RH	Substrate
ROH	Product
ROS	Reactive oxygen species
SFA	Saturated fatty acid
S_2-receptor	Serotonergic receptor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Na-EDTA	Sodium ethylenediaminetetraacetic acid
sGC	Soluble guanylate cyclase
SHR	Spontaneously hypertensive rats
O_2^-	Superoxide anion
SOD	Superoxide dismutase
TXA_2	Thromboxane A_2
TP	Thromboxane A_2 prostanoid receptor
TNF-α	Tumor necrosis factor alpha
3`UTR	3`untranslated region
5`UTR	5`untranslated region
VSMC	Vascular smooth muscle cell
Kv	Voltage-gated K^+ channel
WT	Wild type
WYR	Wistar Kyoto rats
WHO	World Health Organization

Zusammenfassung

Die Prävalenz für Fettleibigkeit ist weltweit gestiegen. Neueste Studien zeigen, dass sogar nach einer Gewichtsreduktion ein erhöhtes Risiko zur Entstehung kardiovaskulärer Erkrankungen besteht. Die Hauptursache für die Entstehung von Fettleibigkeit ist eine fettreiche Ernährung, welche zu einer Gewichtszunahme, Glukoseintoleranz und vaskulären Dysfunktion führen kann. Das Ziel der vorliegenden Dissertation war es, Gene zu identifizieren, deren Expression sich aufgrund einer Diät hohen Fettgehalts ändert und diese Änderung bestehen bleibt nachdem der Fettgehalt der Diät reduziert wurde. In dieser Arbeit wurde ein etabliertes Mausmodell für Diät-induzierte Fettleibigkeit verwendet: Vier Wochen alte, männliche Mäuse des Stammes C57BL/6J wurden entweder für 30 Wochen mit einer Kontrolldiät gefüttert oder mit einer Diät hohen Fettgehalts oder die Mäuse wurden zunächst für 15 Wochen mit der Diät hohen Fettgehalts und abschliessend für 15 Wochen mit der Kontrolldiät gefüttert. Die Verfütterung der Diät hohen Fettgehalts führte nach 30 Wochen zu einer Gewichtszunahme und zu einer verminderten Glukosetoleranz. Es wurden unterschiedliche Auswirkungen auf die durch Phenylephrin-, 5-Hydroxytryptamin und Acetlycholin-induzierte Vasokontraktion in der Thorakalaorta der Mäuse, die mit der Diät hohen Fettgehalts gefüttert wurden als auch in den Mäusen, die beide Diäten erhielten, festgestellt. Eine DNA Exon Microarray Analyse, die mit Aortengewebe (n=3 Replikate pro Gruppe) der drei Mausgruppen durchgeführt wurde, identifizierte nur wenige Gene mit relativ geringen Expressionsunterschieden (< 2.5 fach). Auf Genebene wurden 37 Gene und 46 auf Exonebene gefunden. Die meisten der identifizierten Gene sind an der Regulation der vaskulären Funktion beteiligt. Die Validierung der Gene carbonic anhydrase 3 (*Car3*, Car3) und cytochrome P450, family 2, subfamily E, polypeptide 1 (*Cyp2E1*, Cyp2E1) mittels quantitativer RT-PCR und Western Blot zeigte, dass die Gen- und Proteinexpression durch die Diät hohen Fettgehalts als auch die Verfütterung beider Diäten, herunterreguliert ist. Die Genexpression des Gens cell-death-inducing DFFA-like effector c (*Cidec*) ist durch die Diät hohen Fettgehalts als auch die Verfütterung beider Diäten herunterrguliert wohingegen die Proteinexpression hochreguliert ist. Für das Gen Dipeptidylpeptidase 10 (*Dpp10*) konnte nur gezeigt werden, dass die Genexpression durch die Diät hohen Fettgehalts als auch die Verfütterung beider Diäten, hochreguliert ist. Da *Cyp2E1* die grössten und signifikantesten Expressionsunterschiede (2.2 fach, $p<0.002$) zeigte, wurde es ausführlicher charakterisiert. Auf der Ebene der mRNA Transkripte wurden neben dem vollständigen Transkript zwei neue *Cyp2E1* Spleissformen in der Aorta der Kontrolltiere identifiziert. Die erste Spleissform enthält möglicherweise bis auf einen verkürzten 3`untranslatierten Bereich im Exon 9 die vollständige *Cyp2E1* Nukleotidsequenz. Der zweiten Spleissform fehlen Exon 4-6. Auf der Proteinebene konnte das vollständige Cyp2E1 Protein nicht detektiert werden, aber stattdessen wurden zwei neue Proteinisoformen von Cyp2E1 mit einer Molekulargrösse von 39 und

45 kDa identifiziert. Zudem zeigte die Untersuchung der Gefässreaktivität der Thorakalaorta von Wildtyp und Cyp2E1-defizienten Mäusen, dass Cyp2E1 sowohl eine Rolle in der Regulation der 5-Hydroxytryptamin-induzierten Vasokontraktion als auch in der Vasorelaxation und Vasokontraktion durch Acetylcholin, spielt. Es scheint, dass reaktive Sauerstoffverbindungen und Stickstoffmonoxid zur Regulation beitragen. Zusammenfassend zeigen die Ergebnisse, dass die Verfütterung einer Diät hohen Fettgehalts zu einer Gewichtszunahme, einer Glukoseintoleranz und zu einer Veränderung der Gefässreaktivität führen kann. Zum Teil konnten Veränderungen der Gefässfunktion, die durch die Verabreichung einer Diät hohen Fettgehalts verursacht wurden, nicht durch eine Reduktion des Fettgehalts rückgängig gemacht werden. Zusätzlich legen die Ergebnisse den Schluss nahe, dass Cyp2E1 Spleissformen, die aufgrund der verschiedenen Diäten unterschiedlich exprimiert sind, an der Regulation der vaskulären Funktion beteiligt sein könnten. Zusammenfassend deuten die Resultate darauf hin, dass Cyp2E1 eine zentrale Rolle in der Entstehung von Gefässkrankheiten beim Menschen übernehmen könnte.

Summary

The prevalence of obesity has risen worldwide. Main reason responsible for the development of obesity is intake of nutrition rich in fat. Recent studies suggest that obesity is associated with an increased risk of cardiovascular diseases even after reduction of body weight. Elevated body weight is frequently associated with glucose intolerance and vascular dysfunction. The aim of the present study was to investigate whether alterations in vascular reactivity are associated with changes in steady state mRNA expression levels and gene splicing due to high-dietary fat content and whether these changes sustain after high-dietary fat content is reduced. A well-established mouse model of diet-induced obesity was used. 4 weeks old, male C57BL/6J mice were either fed with a control diet (CD) or a high-fat diet (HFD) for 30 weeks or with a HFD for 15 week followed by a CD for 15 weeks (HFD/CD). Only feeding with HFD increased body weight and led to an impaired glucose tolerance in mice. HFD and HFD/CD showed hetergenous effects on contractile responses induced by phenylephrine, 5-hydroxytryptamine and acetylcholine in thoracic aorta of mice. Subsequent DNA exon microarray analysis using aortic tissue (n=3 replicates/group) from these mice revealed very few genes with relatively small expression changes (<2.5 fold change). At a whole gene level 37 genes and 46 at an exon-specific level were identified. Gene Ontology classification showed that most of the genes are assigned to biological processes involved in regulation of vascular function. mRNA and protein expression levels of the genes carbonic anhydrase 3 (*Car3*, Car3) and cytochrome P450, family 2, subfamily E, polypeptide 1 (*Cyp2E1*, Cyp2E1) were confirmed to be downregulated by HFD and HFD/CD using quantitative RT-PCR and Western Blot, respectively. mRNA expression level of cell-death-inducing DFFA-like effector c (*Cidec*) was downregulated whereas protein expression level was upregulated by HFD and HFD/CD. Dipeptidylpeptidase 10 (*Dpp10*) was confirmed to be upregulated by HFD and HFD/CD only by quantitative RT-PCR. *Cyp2E1* showed highest (fold change of 2.2) and most significant changes ($p<0.002$) in gene expression and therefore was characterized in more detail. On mRNA transcript level, two novel aortic *Cyp2E1* mRNA transcripts were identified in the CD group in addition to full-length *Cyp2E1*. The first transcript possibly consists of the full-length nucleotide sequence with truncated 3`untranslated region in exon 9, while the second transcript lacks exons 4, 5 and 6. On protein level no full-length Cyp2E1 was detectable but two novel aortic Cyp2E1 isoforms at 39 and 45 kDa were identified in all dietary treatment groups. Furthermore, vascular function experiments using thoracic aorta of wild type and Cyp2E1-deficient mice suggest that Cyp2E1 regulates 5-hydroxytryptamine-induced contractions but also acetylcholine-induced relaxation and contraction. Possible mechanisms involve contribution of reactive oxygen species and nitric oxide. In conclusion, the present data show that dietary fat affects body weight, glucose tolerance and vascular reactivity. Some changes in vascular reactivity remained after dietary fat was reduced. Differential expression levels of aortic Cyp2E1 splice

variants during dietary interventions and its role in the regulation of vascular function suggest that this molecule is a novel player in obesity-associated vascular diseases even after fat intake is reduced.

1 Introduction

1.1 Obesity

1.1.1 Statistical data on obesity worldwide

Obesity has become an epidemic and over the last 30 years the prevalence of obesity has steadily increased worldwide (Eckel *et al.*, 2004; Dixon, 2010). The World Health Organization (WHO) monitors the prevalence for obesity worldwide through the Global Database on Body Mass Index (BMI) (Nguyen & El-Serag, 2010). This database contains data of national representative surveys on obesity from countries all over the world. Based on these surveys, a rising trend in obesity is observed in most of the countries despite Denmark and Saudi Arabia in which a decreasing trend was shown (Nguyen & El-Serag, 2010). Data of the WHO showed that in 2005 approximately 1.6 billion people all over the world were overweight and roughly 400 million people were obese (World Health Organization). Prognostic data suggest that by 2015 around 2.3 billion people will be overweight and 700 million will be obese worldwide (Nguyen & El-Serag, 2010). In the USA, 149.3 million people (≥ 20 years of age) were overweight and obese in 2008, which is about 68 % of the population (Roger *et al.*, 2011). In Switzerland, the prevalence for obesity increased from 1992-2007 from 5.4-7.2 % in women and from 8.6-9.7 % in men (Faeh *et al.*, 2011).

1.1.2 Classification of obesity

Increased body fat mass is the main characteristic of obesity (Ogden *et al.*, 2007). According to the WHO, adult overweight and obesity are defined by the BMI which is calculated as weight in kilograms divided by the square of height in meters (kg/m^2) (National Institute of Health (NIH), 1998; Wyatt *et al.*, 2006). A BMI < 18.5 is considered as underweight, a BMI between 18.5 to 24.9 as normal. Persons with a BMI of 25.0 to 29.9 are classified as overweight and with a BMI ≥ 30 as obese. Obesity is further divided into stage I (BMI 30.0-34.9), stage II (BMI 35.0-39.9) or stage III (BMI ≥ 40) obesity (**Figure 1-1**).

A disadvantage of this method is that it does not include factors such as gender, age, ethnicity or composition of the body which can have an impact on accumulation of body fat (Deurenberg *et al.*, 1998). In addition to the BMI measure several other, more direct measures of body fat content exist, namely dual energy x-ray absorptiometry, skinfold thickness, bioelectrical impendance analysis, and underwater weighing (Deurenberg *et al.*, 1998).

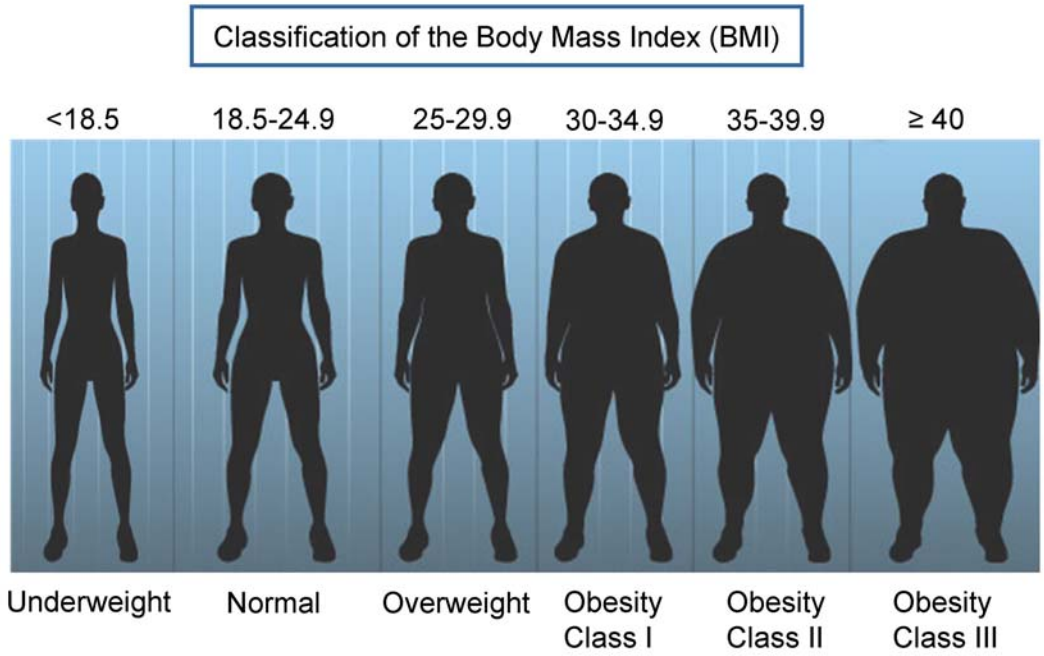

Figure 1-1 Determination of adult Body Mass Index
The Body Mass Index increases with gain in weight and is divided into 5 different classes. Figure adapted from (WebMD).

1.1.3 Overweight and obesity in children and adolescents

The prevalence for childhood and adolescence overweight and obesity has increased enormously in the USA, but also worldwide (Ogden *et al.*, 2002; Jolliffe & Janssen, 2006; Ogden *et al.*, 2010a). Janssen *et al*. determined the percentage of overweight and obese children and adolescents aged 10-16 years in 34 countries including the United States and several European countries in 2001-2002 (Janssen *et al.*, 2005). The study demonstrated that in 26 out of 34 evaluated countries at least 10 % of children and adolescents were overweight and in 7 out of 34 countries, 3 % were obese. Highest prevalence for overweight and obesity was observed in the United States, Great Britain, Greece, Italy, Spain and Malta whereas lowest prevalence was found in Russia and in the Baltic States (Janssen *et al.*, 2005).

BMI in children and adolescents is an age-and gender-dependent BMI (Jolliffe & Janssen, 2006), also referred to as the BMI-for-age, because BMI and waist circumferences change in children and adolescents with growth and maturation (Guo *et al.*, 1997) and varies between girls and boys (Biro *et al.*, 2010). It is calculated as for adults but then compared to reference values in form of percentiles typical for children of same age and gender. A BMI-for-age < 5^{th} percentile defines underweight, 5^{th}-84^{th} percentile is due to normal weight, ≥ 85^{th} percentile but < 95^{th} percentile defines overweight and a BMI-for-age of ≥ 95^{th} percentile reflects obesity (Barlow, 2007). It is widely known that childhood and adolescent obesity is associated with several different cardiovascular risk factors mainly type 2 diabetes (Fagot-Campagna *et al.*, 2000),

hypertension (Genovesi *et al.*, 2005), and the metabolic syndrome (Cook *et al.*, 2003). Additionally, obesity in children and adolescents is a predictor for the development of cardiovascular diseases in adulthood (Baker *et al.*, 2007). The probability that overweight children and adolescents become obese in adulthood is high, particularly with increasing BMI (Guo & Chumlea, 1999) or if their parents are obese as well (Whitaker *et al.*, 1997). Must and colleagues showed that children and adolescents who were obese had a high risk to develop cardiovascular diseases as adults even if they lost weight during adulthood (Must *et al.*, 1992). Therefore, a lot of effort should be put in prevention of childhood and adolescent obesity to decrease the risk for cardiovascular illnesses in adulthood. Most important preventive steps include regular physical activity and a healthy nutrition in everyday life of young people. Fat intake, carbohydrate, protein intake, and the amount of food eaten every day should be adapted to the age (Daniels *et al.*, 2005). Family, schools and the community should be involved in preventive steps.

1.2 Health burden of obesity: Causes, consequences, and costs

1.2.1 Causes of obesity

Factors contributing to the development of obesity are diverse and complex. Common causes are increased energy intake by nutrition high in fat content, sedentary lifestyle, as well as genetic, social and environmental factors (Wyatt *et al.*, 2006; Nguyen & El-Serag, 2010). Not only in the United States high energy intake is promoted in terms of cheap, high energy-dense food ("fast food"), soft drinks and availability in huge amounts (Hill *et al.*, 2003), but also in many developing countries a trend in this direction is observed (Popkin, 2001). Urbanization and westernization more and more replace food high in complex carbohydrates and fibres by food high in sugar and fat (Drewnowski, 2000; Popkin, 2001). One major problem is that energy intake in terms of excess in food increases but energy expenditure in form of physical activity decreases. Sedentary activities as watching television and surfing in the internet increase (Hill *et al.*, 2003; Nguyen & El-Serag, 2010). Dietz and Gortmaker showed that each additional hour of watching television per day increases the prevalence of obesity about 2 % (Dietz & Gortmaker, 1985). Genetic predisposition is an additional factor involved in the development of obesity, but not many genes are known until now that can be associated with obesity. Single gene mutations in the leptin receptor, melanocortin-4-receptor, pro-opiomelanocortin, and leptin gene play a role in the development of obesity (Andreasen & Andersen, 2009). Frayling *et al*. showed that single nucleotide polymorphisms in the fat mass and obesity gene are associated with increased BMI and type 2 diabetes (Frayling *et al.*, 2007). Several other research laboratories confirmed this finding (Andreasen *et al.*, 2008; Haupt *et al.*, 2008; Hunt *et al.*, 2008). Social aspects in terms of obesity are important to consider, too. Obese people often have less social networks and are discriminated by society (Puhl & Brownell, 2001). With lower

educational classifications and social status higher prevalence of obesity is observed (Ogden *et al.*, 2010b). Studies showed that a higher household income improves the quality of food (Drewnowski & Specter, 2004). Environmental factors such as industrialization of countries, more jobs in which less physical activities are needed, the food industry which advertises unhealthy eating habits increased the prevalence for obesity in the society. People are not willing anymore to invest much effort and time to cook because convenient food is relatively cheap and easily accessible (Wyatt *et al.*, 2006). Other data indicate that the average caloric intake per day/per person has risen over the years (Nestle & Jacobson, 2000). All those different factors can have a significant impact on a person's risk to become obese.

1.2.2 Consequences of obesity

Obesity is associated with risk of mortality. People with a BMI greater than 30 are highly endangered to die earlier than non obese people (Calle *et al.*, 1999; Dixon, 2010). Annual number of deaths related to obesity range between 110000 and 400000 (Flegal *et al.*, 2005; Olshansky *et al.*, 2005). Other consequences of obesity include the metabolic syndrome, type 2 diabetes, and hypertension, which can lead to cardiovascular diseases (Field *et al.*, 2001; Klein *et al.*, 2004a).

1.2.2.1 Obesity and cardiovascular disease

One of the major risk factors for cardiovascular diseases is obesity (Abate, 2000; Yusuf *et al.*, 2004). Cardiovascular diseases kill approximately 17 million people per year worldwide (World Health Organization). These diseases affect the heart and blood vessels. The most important known cardiovascular diseases are atherosclerosis, angina pectoris, cerebrovascular disease (main cause of stroke), coronary artery disease, peripheral arterial disease, and congestive heart failure (Poirier *et al.*, 2006). The risk for cardiovascular morbidities is higher in obese people compared to lean individuals (Van Gaal *et al.*, 2006). Since obesity is associated with promoting the development of insulin resistance, type 2 diabetes, metabolic syndrome and hypertension, the interaction of all these metabolic alterations and abnormalities contribute to the progression of cardiovascular diseases. The molecular mechanisms by which obesity causes cardiovascular diseases are still not completely understood and are therefore investigated intensely by researchers all over the world. A main characteristic of obesity is an increased waist circumference indicative of accumulation of abdominal or visceral fat. Visceral fat is mainly composed of adipocytes (Ibrahim, 2010) which synthesize and secrete several pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-6, and acute-phase protein C-reactive protein (CRP). Levels of inflammatory cytokines are frequently increased in obese people (Grundy, 2004). Adipocyte-derived hormones (adipokines) such as adiponectin, leptin and resistin, plasminogen activation inhibitor-1 (PAI-1), and nonesterified fatty acids (NEFAs) (Heptulla *et al.*, 2001; Scherer, 2006; Shoelson *et al.*, 2006) are additional

molecules secreted by adipocytes (Ritchie & Connell, 2007). Adiponectin is a hormone exclusively secreted by adipose tissue which regulates glucose and fatty acid metabolism. It is known to be anti-inflammatory and anti-diabetic. In obese patients, levels of adiponectin are usually low. Leptin on the other hand is a hormone also released from adipose tissue which suppresses food intake (Friedman & Halaas, 1998) by binding to receptors in the hypothalamus (Elmquist *et al.*, 1999). However, leptin levels are elevated in obesity which can be due to leptin resistance caused by binding of CRP to leptin resulting in an inhibition of leptin receptor signaling (Chen *et al.*, 2006). CRP is increased and secreted by the liver in response to adipokines (Pepys & Hirschfield, 2003). Furthermore, serum resistin, a cysteine-rich protein, is elevated in obesity. It is known to be highly expressed in adipocytes and is strongly associated with insulin resistance (Degawa-Yamauchi *et al.*, 2003). PAI-1 inhibits fibrinolysis which is usually increased in obesity and is associated with an increased risk for thrombosis (Grundy, 2004). It can be secreted by liver, vascular endothelium, and adipose tissue. An accepted explanation is that PAI-1 derived from adipose tissue is activated via inflammatory cytokines such as TNF- α (De Taeye *et al.*, 2005). Obesity increases accumulation of triglycerides in visceral fat. Triglycerides can be cleaved to glycerol and NEFAs by the hormone sensitive lipase, a process called as lipolysis (Macfarlane *et al.*, 2008) leading to an increase of NEFAs in the blood (Heptulla *et al.*, 2001). Furthermore, these elevated levels of NEFAs increase triglyceride storage in the liver (Grundy, 2000) promoting the development of atherogenic dyslipidemia. It is characterized by high levels of triglycerides and hypercholesterolemia (elevated levels of low density lipoprotein (LDL) and low high density lipoprotein (HDL) cholesterol levels). Atherogenic dyslipidemia is a metabolic risk factor strongly correlated with the metabolic syndrome which is linked to the development of cardiovascular diseases (Ritchie & Connell, 2007). Taken together, obesity increases the amount of visceral fat mass which leads to alterations in the production of adipocyte-derived inflammatory markers, adipokines and nonesterified free fatty acids. These changes can either independently affect the vasculature or lead to insulin resistance resulting in type 2 diabetes and hypertension or other diseases thereby causing an impact on the vasculature by decreasing nitric oxide (NO) availability and increasing the generation of reactive oxygen species (ROS). This leads to endothelial dysfunction, a pre-stage for the development of cardiovascular diseases such as atherosclerosis (**Figure 1-2**). Obesity promotes the onset of atherosclerosis, a chronic inflammatory disease of arteries in which accumulation of fat deposits (plaques) leads to thickening of the arterial wall. This consequence can disturb normal blood flow and sudden rupture of plaques can lead to atherothrombosis occluding the whole vessel lumen (Thim *et al.*, 2008). Consequences are heart attacks (myocardial infarction) (Kubo *et al.*, 2007) and strokes in the brain (ischemic stroke) (Spagnoli *et al.*, 2004).

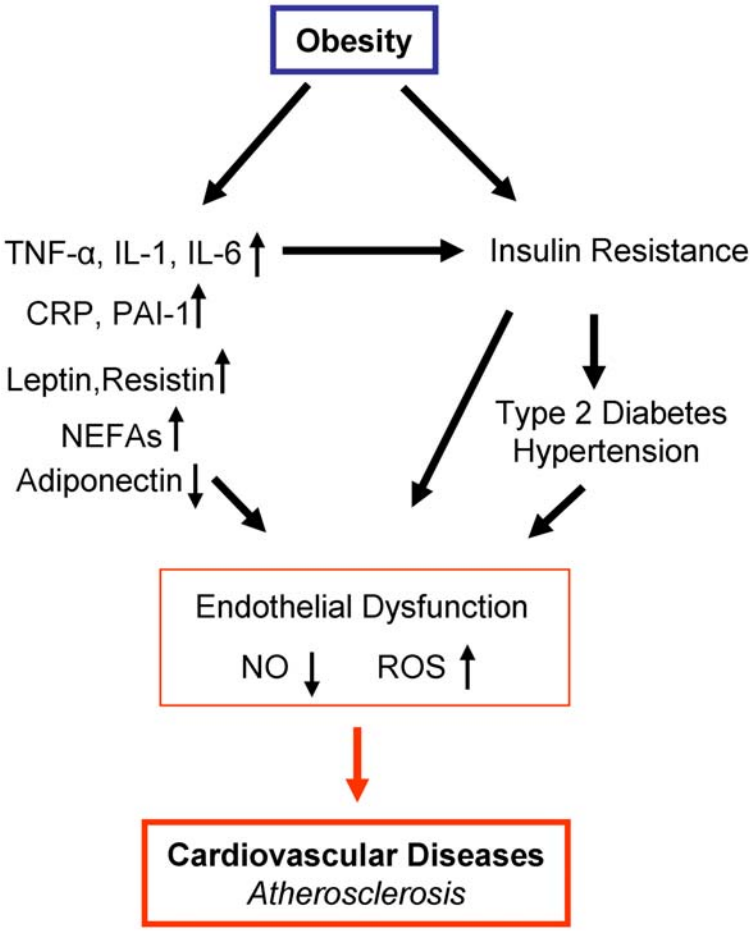

Figure 1-2 Mechanism linking obesity to cardiovascular diseases
TNF-α, tumor-necrosis factor-alpha; IL-1, interleukin-1; IL-6, interleukin-6; CRP, C-reactive protein; PAI-1, plasminogen activation inhibitor-1; NEFAs, nonesterified free fatty acids; NO, nitric oxide; ROS, reactive oxygen species; ↑ increase**;** ↓ decrease. Adapted from (Ritchie & Connell, 2007).

1.2.2.2 Obesity-associated burden and costs on economy

Obesity is not only a risk factor for several diseases and death, but is an increasing financial burden for the economy (Nguyen & El-Serag, 2010). Costs for obesity-related health problems are immense due to treatments and increase in professional nursing staff (Friedman & Fanning, 2004). Obesity-associated expenses by health programs in the USA increased from 6.5 % in 1998 to 9.1 % in 2008 (Finkelstein *et al.*, 2009). Half of these costs are paid by Medicaid and Medicare, which are American governmental health-programs covering health and medical services for disabled people and families with low income (Friedman & Fanning, 2004). Over the last 35 years, costs for annual health care have risen dramatically for aged obese patients (Wyatt *et al.*, 2006). Thorpe

et al. investigated how obesity-associated diseases influence private health care expenditures between 1987 and 2002. The results showed that private health care costs enhanced from 2 % to 11.6 % which accounts for a rise from $3.6 billion to $36.5 billion US dollars indicating an increase of privately insured obese people (Thorpe *et al.*, 2005). In Switzerland about 7.3 % of total health care expenses were spend on overweight-or obesity-related diseases in 2006. Expenses on only obesity-related costs were approximately 0.38 % of the total gross domestic product in 2006 (Eidgenössisches Bundesamt für Gesundheit, 2009).

1.2.3 Treatment of obesity

Moderate reduction of weight (mainly reduction in visceral adipose tissue) has beneficial effects on cardiovascular diseases (Orzano & Scott, 2004). Weight loss reduces the risk for type 2 diabetes (Laaksonen *et al.*, 2005), hypertension (Mertens & Van Gaal, 2000), the metabolic syndrome (Gill & Malkova, 2006), and improves endothelial dysfunction (Ziccardi *et al.*, 2002). Main approaches to achieve weight loss are physical activity, changing of food habits, pharmacological and surgical treatments (Ritchie & Connell, 2007). Already changes in life style can reduce the risk of development of type 2 diabetes up to 60 % and the metabolic syndrome up to 37 % (1998). Furthermore, LDL cholesterol and triglycerides are decreased and HDL cholesterol increases (Van Gaal *et al.*, 1997). Commonly used drugs in the treatment of obesity are sibutramine (Filippatos *et al.*, 2005), orlistat (Sjostrom *et al.*, 1998), and rimonabat (Van Gaal *et al.*, 2005). Treatment with all three drugs results in weight reduction and improvement of cardiovascular risk factors. Sibutramine stimulates noradrenergic and serotonergic activity by reducing appetite (Van Gaal *et al.*, 1998). It increases HDL-cholesterol levels, but side effects such as increase in blood pressure (Atkinson, 1997) and heart rate (Zannad *et al.*, 2002) can occur. Orlistat inhibits lipase thereby reducing fat absorption. It decreases mostly LDL-cholesterol, improves insulin sensitivity and fasting glucose (Swinburn *et al.*, 2005). Orlistat is not only approved in the treatment of adult obesity but also in childhood obesity (Norgren *et al.*, 2003). Rimonabat, a cannaboid receptor-1 inhibitor, is effectively used in the reduction of weight in obese patients resulting in increased HDL-cholesterol and insulin sensitivity (Van Gaal *et al.*, 2005). Extreme obesity with a BMI > 40 kg/m^2 is treated by bariatric surgery in which the stomach of obese patients is reduced by implanting a gastric band. It is a very efficient method to reduce weight and visceral adipose fat combined with improvements in cardiovascular risk factors (Hanusch-Enserer *et al.*, 2004). This is controversial since studies have shown that in extreme obese individuals, who lost weight after gastric banding, the risk for cardiovascular diseases did not decrease (Busetto *et al.*, 2004; Klein *et al.*, 2004b). The duration of fat intake over a long period has negative effects on the body metabolism and often the body is not able to reverse these effects (Murphy *et al.*, 2006). Brook *et al*. showed that weight loss over 3 months reduced some metabolic factors but conduit artery function did not improve (Brook *et*

al., 2004). Additionally, Skilton and coworkers confirmed that short-term weight loss is associated with improvements in some metabolic mediators but did not improve cardiac and vascular functions (Skilton *et al.*, 2008). Maintaning weight is an important criterion to improve cardiovascular risk factors (Wadden *et al.*, 1999). Regaining of weight after weight reduction is associated with an increase in cardiovascular risk factors (Hensrud *et al.*, 1995). Important to note is that pharmacological and surgical approaches should only be considered if changes in nutrition and physical activity have failed (Flodmark *et al.*, 2004).

1.3 The circulatory system

1.3.1 Organization and function of the circulatory system

The circulatory system transports blood cells, oxygen and carbon dioxide, nutrients, hormones, and waste products through the body via the vascular system which is composed of several types of blood vessels. This system is needed to maintain body homeostasis. It can be further divided into the cardiovascular and lymphatic systems. The cardiovascular system includes the heart, a network of vessels (arteries, veins and capillaries) and blood whereas the lymphatic system is composed of lymph nodes, lymph vessels and lymph (Pugsley & Tabrizchi, 2000). The cardiovascular system will be discussed in more detail. The pulmonary circulation and the systemic circulation form the cardiovascular system. Part of the pulmonary circulation are the right heart (right atrium and ventricle), the lungs and deoxygenated blood. The systemic circulation is composed of the left heart (left atrium and ventricle), systemic organs, and oxygenated blood. The heart is the main organ responsible to pump the blood into the arterial system via the aorta, arterioles, and capillaries to provide organs with oxygenated blood as well as returning deoxygenated blood through the venous system via venules and veins back to the heart (Mohrman & Heller, 2010). This process is repeated and called the circulation cycle. **Figure 1-3** summarizes the circulation cycle.

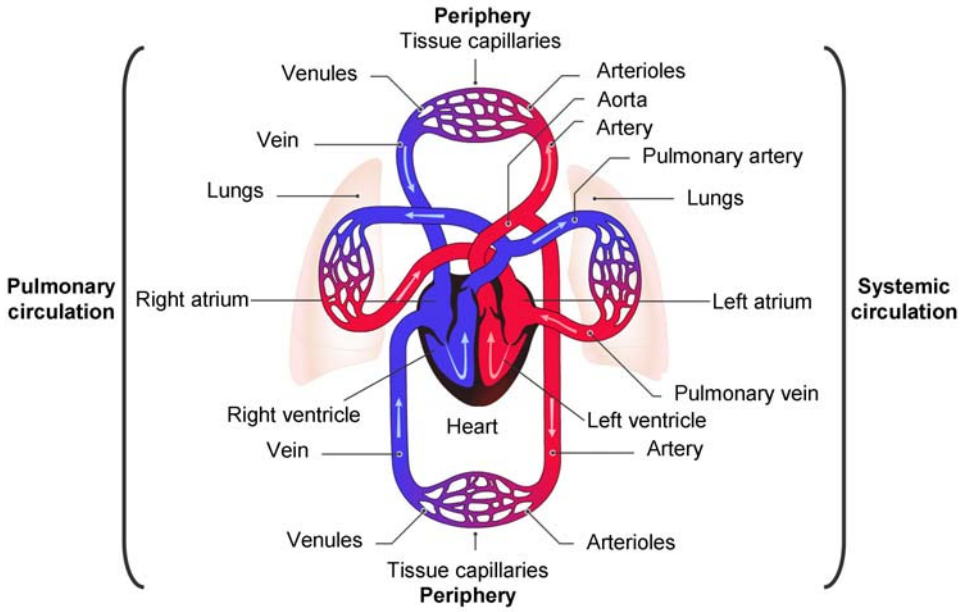

Figure 1-3 The circulation cycle of the human cardiovascular system
The cycle begins with the blood (deoxygenated) coming back from the organs and entering the right atrium of the heart. Then, it passes into the right ventricle followed by pumping the blood into the lungs in which the blood is oxygenated (pulmonary circulation). Oxygenated blood flows from the lungs into the left atrium of the heart to be further pumped into the left ventricle. From there, blood passes into the aorta which supplies the periphery of the body with oxygenated blood (Systemic circulation). Oxygenated and deoxygenated blood is illustrated in red and blue, respectively. Arrows indicate the direction of the blood flow. Figure adapted from (UrgoMedical).

1.3.2 Types of blood vessels

Blood vessels are divided into arteries (aorta, arterioles, and capillaries) and veins (venules, vein). Their main function is to transport oxygenated blood from the left heart to organs of the body and to return deoxygenated blood back to the heart. These vessels differ in vessel diameter and wall thickness. The aorta is the largest artery and consists of an internal diameter of 25 mm in men. It is divided into five sections: aortic root, ascending aorta, aortic arch, descending thoracic aorta, and abdominal aorta (**Figure 1-4**). It is often referred to as conduit artery because its resistance to blood flow is low (Greenwald, 2007). Smaller vessels are arterioles with an internal diameter of 30 µm. They are referred to as resistance arteries because resistance to blood flow is very variable. Capillaries, with an internal vessel diameter of 5 µm, build the smallest group of vessels in the arterial system. Total surface of capillaries in the human body is around 1000 m^2. When blood flows through capillaries, an exchange of nutrients and oxygen between organs and blood happens. Therefore, these vessels are often called as exchange vessels. After the blood has flown through capillaries, it enters venules and veins to be returned back to the heart. They are involved in pumping back deoxygenated blood upwards of the body into the right heart. For this purpose, venules and veins

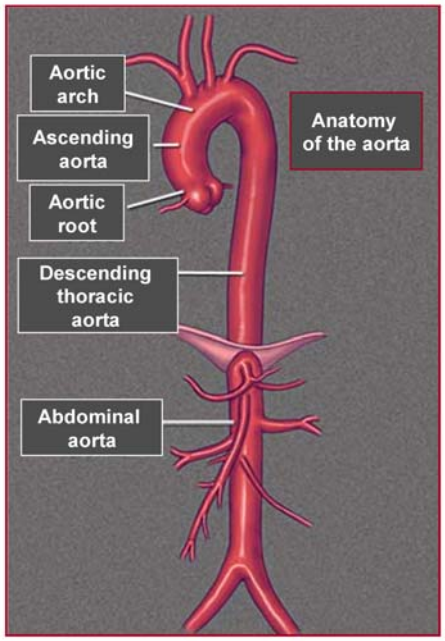

Figure 1-4 Anatomical structure of the aorta
The aorta is divided into five distinct regions: aortic root, ascending aorta, aortic arch, descending thoracic aorta, and abdominal aorta. Figure adapted from (MayoClinic).

contain one-way valves which avoid the back flow of the blood (Borysenko & Beringer, 1984; Mohrman & Heller, 2010).

Arteries are classified in elastic (conducting) and muscular (distributing) arteries (Borysenko & Beringer, 1984). Aorta, carotid artery, and renal artery are elastic arteries because of the huge amount of elastic fibres. This is an important feature to transport large blood volumes forward. Most arteries and veins are of muscular nature. These arteries consist of large layers of vascular smooth muscle cells (VSMCs) and less elastic fibres. (Pugsley & Tabrizchi, 2000) Arterioles, capillaries, venules and veins belong to this type of arteries. Muscular arteries play a crucial role in distributing the blood to the periphery (Golenhofen, 2000; Mohrman & Heller, 2010).

1.3.3 Structure of the blood vessel wall

Blood vessels are histological divided into three different layers termed *tunica intima*, *tunica media* and *tunica adventitia* (Pugsley & Tabrizchi, 2000). The *tunica intima* is the innermost layer containing a single layer of endothelial cells (ECs) which are exposed to the blood flowing through the lumen of the vessel. Additional layers are the subendothelium with fibro-elastic connective tissue and a layer of elastic lamina that stabilizes endothelial cells and provide flexibility. The *tunica media* consists of multiple layers of VSMCs, elastin fibres and external elastic lamina. The third layer, called *tunica adventitia*, is the outermost mainly containing fibro-elastic connective tissue (collagen) and fibroblasts (**Figure 1-5**). Usually, big arteries and veins are surrounded

by the *vasa vasorum*, a network of small vessels, supplying large vessels with oxygen and nutrients (Borysenko & Beringer, 1984; Mohrman & Heller, 2010).

One common characteristic of all types of vessels is the single layer of endothelial cells. Arteries have a "thick" wall because the *tunica media* contains besides multiple layers of VSMCs, a lot of elastin and collagen fibres. The *tunica media* of venules and veins is "thinner" than in arteries, because of fewer amounts of VSMCs and elastic fibres. Veins are more distensible, because of their "thinner" walls.

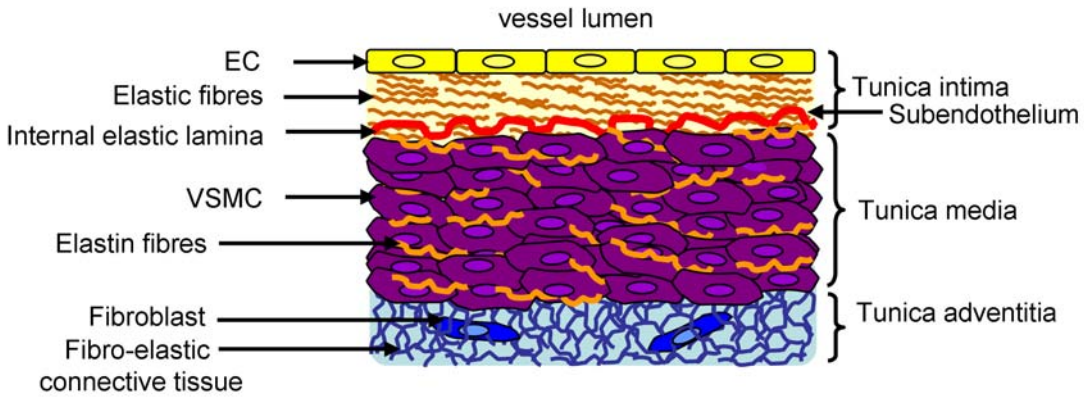

Figure 1-5 Anatomy of a blood vessel wall
An artery is structured into three distinct layers: The *tunica intima*, *tunica media*, and *tunica adventitia*. The *tunica intima* contains endothelial cells (ECs). The subendothelium with the interna elastic lamina and elastic fibres also is referred to the *tunica intima*. Mainly vascular smooth muscle cells (VSMCs) and elastin fibres are present in the *tunica media*. The third layer named *tunica adventitia* consists of fibro-elastic connective tissue in which fibroblasts are embedded.

1.3.3.1 Endothelial cells

ECs form a tight monolayer called endothelium. It represents the interface between lumen and the rest of the vessel wall. ECs are involved in diverse functions. The endothelium is permeable for different nutrients, biological active macromolecules, and blood cells such as leukocytes. Additionally, ECs play an important role in vasculogenesis, angiogenesis, regulation of vascular tone, pro-and anti coagulation, and inflammation (Cines *et al.*, 1998). Development of vascular ECs is termed vasculogenesis (Risau & Flamme, 1995) which begins in the embryonic state. Angiogenesis is defined as sprouting of ECs to expand the vascular network from already existing vessels (Sato *et al.*, 1995). Regulation of vascular tone is mediated by ECs via the release of various vasoactive molecules having vasodilator or vasoconstrictor functions (Cines *et al.*, 1998). NO is a potent vasodilator secreted from ECs(Mulvany, 1993). Other functions of NO include inhibition of VSMC migration (Marks *et al.*, 1995) and proliferation (Garg & Hassid, 1989), platelet aggregation, expression of adhesion molecules on ECs, endothelin-1 production, oxidation of LDL, and adhesion of monocytes and platelets (Michel & Vanhoutte, 2010). An additional

important function of ECs is to keep a balance between antithrombotic and prothrombotic mechanisms. Under normal conditions an antithrombotic state is present in ECs whereas during injury of ECs prothrombotic properties take over (Cines *et al.*, 1998). The role of ECs in inflammation is pivotal. The expression of endothelial cellular adhesion molecules stimulate leukocytes and macrophages to roll, adhere and migrate through them to reach the inflammatory source (Cines *et al.*, 1998).

1.3.3.2 Vascular smooth muscle cells

The flexibility of VSMCs to modify their phenotype depending on normal physiological and pathological conditions is essential to keep vascular homeostasis (Owens, 1995). Under normal condition, VSMCs are in a differentiated state and mainly located in the medial layer. These cells express receptors, channels, regulatory and contractile proteins important for the regulation of vascular tone. This is crucial to regulate blood vessel diameter by contraction and dilation to maintain blood flow and blood pressure (Owens *et al.*, 2004). Migratory and proliferative properties as well as the generation of extracellular matrix are decreased in differentiated VSMCs (Owens, 1995). The most important differentiation or maturation markers are smooth muscle α-actin (Fatigati & Murphy, 1984), smooth muscle myosin heavy chain (Aikawa *et al.*, 1993), calponin (Nishida *et al.*, 1993), smooth muscle-22 α (Nishida *et al.*, 1993), caldesmon (Sobue & Sellers, 1991), and smooth muscle α-tropomyosin (Ruiz-Opazo & Nadal-Ginard, 1987). VSMCs are dynamic cells able to switch phenotype from a differentiated to a dedifferentiated state which is associated with changes in structure and function of the cells. This often occurs during different developmental stages of the vessel, vascular injury and is often found in vascular diseases such as atherosclerosis (Owens *et al.*, 2004) and hypertension. An early step of atherosclerosis development is phenotypic switching of VSMCs from a differentiated into a dedifferentiated state in which the cells loose their contractile properties and which allows the cells to migrate from the *tunica media* into the *tunica intima* where VSMCs produce and secrete for example inflammatory cytokines (Campbell & Campbell, 1994; Owens *et al.*, 2004). In a dedifferentiated state, VSMCs are highly migratory, proliferative and synthesis of extracellular matrix is increased (Owens *et al.*, 2004).

1.3.4 Mechanisms of vasodilatation

ECs in the blood vessel wall are exposed to blood flow and play a pivotal role in regulating vascular tone by mediating vascular relaxation or contraction of VSMCs (Vanhoutte *et al.*, 2009).

One of the most important mechanisms how vasodilatation is mediated was described by Furchgott and Zawadzki in 1980. These authors showed that acetylcholine causes endothelium-dependent relaxation by release of a vasodilator molecule termed endothelium-dependent relaxing factor (EDRF) (Furchgott & Zawadzki, 1980). This important EDRF was later found to be NO (Ignarro *et al.*, 1987). Robert F. Furchgott,

together with Louis J. Ignarro and Ferid Murad, received the Noble price in Medicine for "NO as a signaling molecule in the cardiovascular system" in 1998 (The Official Web Site of the Nobel Prize). Acetylcholine causes endothelium-dependent relaxation by stimulating the release of NO but also other substances such as adenosine diphosphate (ADP), bradykinin, thrombin, and prostacyclin (PGI_2) bind to specific receptors on endothelial cells triggering relaxation of VSMCs (Lüscher & Vanhoutte, 1990). NO is the most potent vasodilator and has a protective role against endothelial dysfunction and vascular diseases (Li & Forstermann, 2000). NO and L-citrulline are produced by converting L-arginine via endothelial NO synthase (eNOS or NOS III). eNOS activity is dependent on calcium (Ca^{2+}), calmodulin, nicotinamide adenine dinucleotide phosphate (NADPH), oxygen (O_2), and the important cofactor (6*R*) 5, 6, 7, 8-tetrahydro-L-biopterine (BH_4) (Forstermann & Munzel, 2006). Besides eNOS which is exclusively expressed in ECs, two other NO synthases are described called neuronal NOS (nNOS or NOS I) expressed in central and peripheral neuronal cells and inducible NOS (iNOS or NOS II) first found in macrophages of mice. eNOS is the most important enzyme amongst the other two in the vasculature, but nNOS and iNOS can also act on the vasculature, mainly in pathophysiological conditions (Li & Forstermann, 2000). Activation of eNOS begins with binding of acetylcholine to M_3-muscarinic receptors stimulating phospholipase C (PLC) which leads to the formation of inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4, 5-bisphosphate (PIP_2). IP_3 enhances Ca^{2+} influx through Ca^{2+} channels leading to an increase in intracellular Ca^{2+}. Further, Ca^{2+} induces activation of calmodulin which binds to eNOS resulting in eNOS activity and thus generation of NO and citrulline from L-arginine. NO diffuses from ECs to VSMCs and activates soluble guanylate cyclase (sGC) leading to production of cyclic guanosine 3`, 5`-monophosphate (cGMP) from guanosine-5'-triphosphate (GTP). Protein kinase G (PKG) is activated by cGMP decreasing intracellular Ca^{2+} in VSMCs which causes vasodilatation (**Figure 1-6**) (Avogaro & de Kreutzenberg, 2005). Besides NO, PGI_2, belonging to the group of vasodilator prostaglandins, can be released by ECs leading to NO generation and relaxation of VSMCs by increase in cyclic 3`, 5`-adenosine monophosphate (cAMP). Increase of eNOS activity and thus release of NO leading to vasorelaxation can be initiated by several, different factors such as shear stress (Yan *et al.*, 2007), estrogens (Scott *et al.*, 2007), adiponectin (Zhu *et al.*, 2008), insulin (Fisslthaler *et al.*, 2003), and aldosterone. A decrease in eNOS activity reduces NO bioavailability leading to impairment of vasodilatation which can be caused by oxygen-derived free radicals (Vanhoutte *et al.*, 2009), ageing (Bulckaen *et al.*, 2008), obesity (Avogaro & de Kreutzenberg, 2005), hypercholesterolaemia (Aubin *et al.*, 2008), hypertension (Damjanovic & Barton, 2008), and diabetes (Vanhoutte *et al.*, 2009)

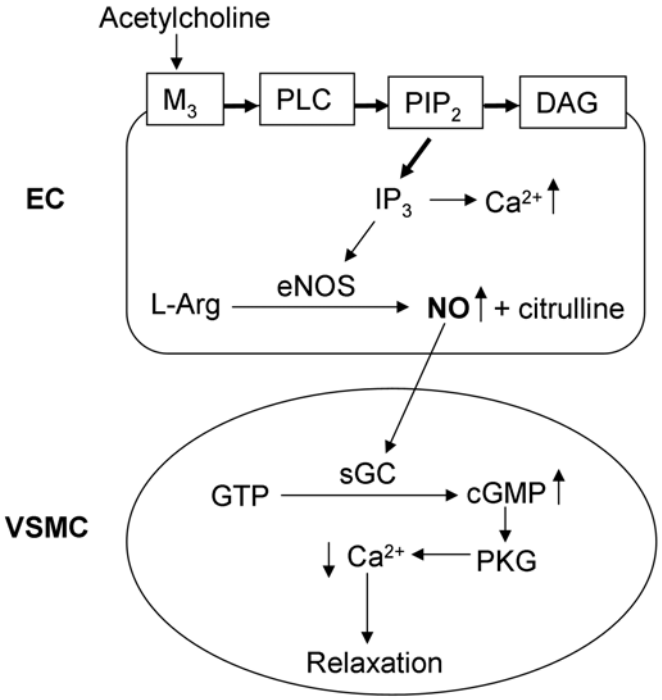

Figure 1-6 Endothelium-dependent relaxation
Activation of M_3-muscarinic receptor on endothelial cells by acetylcholine activates PLC leading to formation of DAG and IP_3 from PIP_2. IP_3 increases intracellular Ca^{2+} which activates eNOS to generate NO. NO diffuses from endothelial cells to vascular smooth muscle cells activating sGC which leads to an increase in cGMP. Then, PKG is stimulated decreasing intracellular Ca^{2+} resulting in vasorelaxation of VSMCs. EC, endothelial cell; VSMC, vascular smooth muscle cell; M_3, muscarinic receptor; PLC, phospholipase C; PIP_2, phosphatidylinositol 4, 5-bisphosphate; DAG, diacylglycerol, IP_3, inositol 1, 4, 5-triphosphate; L-Arg, L-arginine; eNOS, endothelial nitric oxide synthase, NO, nitric oxide; GTP, guanosine-5'-triphosphate; sGC, soluble guanylate cyclase; cGMP, cyclic guanosine 3`, 5`-monophosphate; PKG, protein kinase G. Adapted from (Avogaro & de Kreutzenberg, 2005).

Endothelium-dependent relaxation is not only mediated by NO and PGI_2 by activating cGMP and cAMP, respectively but also by endothelium-dependent hyperpolarizing factors (EDHFs) which initiate relaxation by hyperpolarization of VSMCs (Feletou & Vanhoutte, 2009). Known EDHFs are carbonic monoxide (CO), hydrogen sulfide, hydrogen peroxide (H_2O_2), and cyclooxygenase (COX), lipooxygenase (LOX) or cytochrome P450 mono-oxygenases-derived metabolites. Hyperpolarization of the underlying VSMCs is mediated by activation of potassium (K^+) channels through these factors resulting in vasodilatation (Feletou & Vanhoutte, 2009). Interestingly, in some arteries, NO and PGI_2 act as EDHFs by directly hyperpolarizing VSMCs through activation of K^+ channels (Bolotina *et al.*, 1994; Parkington *et al.*, 2004). Furthermore, vasodilator responses to NO occur more frequently in larger vessels whereas EDHFs are released in smaller vessels (Avogaro & de Kreutzenberg, 2005).

1.3.5 Mechanisms of vasoconstriction

ECs can also release contractile factors, called endothelium-derived contracting factors (EDCFs) causing vasoconstriction of VSMCs (Vanhoutte & Tang, 2008). Mainly, prostanoids generated by endothelial COX are responsible for endothelium-dependent contractions. Two COX isoforms, COX-1 and COX-2, exist. Experiments with COX-1-deficient and COX-2-deficient mice revealed that mainly COX-1 mediates prostanoid-evoked endothelium-dependent contractions (Tang *et al.*, 2005). Other studies showed that endothelium-dependent contractions can also be mediated by COX-2 (Shi *et al.*, 2008). These contractions can be blocked by addition of non-selective COX inhibitors, such as meclofenamate and indomethacin (Sanchez-Ferrer & Marin, 1990; Woods *et al.*, 2001; Traupe *et al.*, 2002). A reduction in NO availability either through inhibition of NO synthase or pathophysiological conditions such as obesity or hypertension favor endothelium-dependent contrations (Vanhoutte, 2009; Vanhoutte *et al.*, 2009). Production of prostanoids or EDCFs is mediated by acetylcholine or ADP which binds to M3-muscarinic receptors or P2y purinoceptors on ECs, respectively (Vanhoutte, 2009). This leads to an increase in intracellular Ca2+ and activation of phospholipase A2 (PLA2) which acts on arachidonic acid (AA) in a way that COX metabolizes it to produce endoperoxides (Vanhoutte & Tang, 2008). These endoperoxides can cause vasoconstriction by activation of VSMCs (Vanhoutte *et al.*, 2005) or they are converted further into prostanoids such as PGI2, thromboxane A2 (TXA2), prostaglandin D2 (PGD2), prostaglandin E2 (PGE2), and prostaglandin F2alpha (PGF2α) (Bos *et al.*, 2004; Norel, 2007) involved in evoking endothelium-dependent contractions (**Figure 1-7**). Inhibition of TXA2 prostanoid receptors (TP) on VSMCs showed that cyclooxygenase-dependent prostanoids bind to these receptors leading to endothelium-dependent contractions(Yang *et al.*, 2003; Vanhoutte & Tang, 2008).

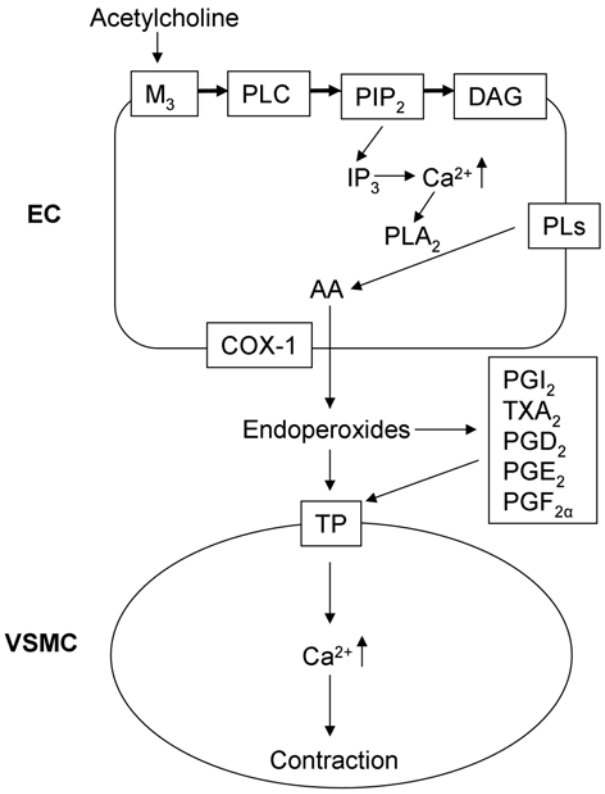

Figure 1-7 Endothelium-dependent contraction
Acetylcholine binds to the muscarinic receptor on endothelial cells and activates PLC leading to formation of DAG and IP_3 from PIP_2. IP_3 increases intracellular Ca^{2+} which stimulates PLA_2 to activate the release of AA from PLs. Then AA is metabolized by COX-1 to endoperoxides which itself can cause endothelium-dependent contraction by binding to TP receptors on VSMCs or by conversion to other vasoconstrictor prostaglandins (PGI_2, TXA_2, PGD_2, PGE_2, $PGF_{2\alpha}$). EC, endothelial cell; VSMC, vascular smooth muscle cell; M_3, muscarinic receptor; PLC, phospholipase C; PIP_2, phosphatidylinositol 4, 5-bisphosphate; DAG, diacylglycerol, IP_3, inositol 1, 4, 5-triphosphate; PLs, phospholipids, PLA_2, phospholipase A_2, AA, arachidonic acid, COX-1, cyclooxygenase-1; PGI_2 prostacyclin; PGI_2, TXA_2, thromboxane A_2; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; TP, TXA_2 - selective prostanoid receptor. Adapted from (Vanhoutte & Tang, 2008).

Another important vasoconstrictor is 5-hydroxytryptamine. It is a neurotransmitter and monoamine derived from tryptophan (Horn, 2003) which is known to play an important role in cardiovascular function since decades (Page, 1958). It has vasoconstrictor as well as vasodilator properties *in vivo*, whereas *in vitro* it causes vasoconstriction in most isolated large blood vessels (Van Nueten *et al.*, 1985). 5-hydroxytryptamine-induced contractions are mainly mediated by activation of serotonergic (S_2) receptors on VSMCs (Vanhoutte *et al.*, 1984) which can be inhibited by serotonergic antagonists such as ketanserin, spiperone, or methysergide (Van Nueten *et al.*, 1985). Whether 5-hydroxytryptamine leads to vasoconstriction or vasodilatation depends on the balancing effect of various factors. Additionally, sensitivity of 5-hydroxytryptamine differs between various vascular beds (Vanhoutte *et al.*, 1984). Russel *et al.* showed that the EC50 (concentration at which a drug causes a half-

maximal effect) for 5-hydroxytryptamine in thoracic aorta of C57BL/6J mice is ~ 0.1 μM (Russell & Watts, 2000) whereas in several different canine arteries EC50 values for 5-hydroxytryptamine varied from ~ 0.01 μM-1 μM (Van Nueten *et al.*, 1985). Contractions are dependent on the influx of intracellular or extracellular Ca^{2+} depending on the blood vessel (Bolton, 1979; Van Nueten *et al.*, 1985). An interesting feature of 5-hydroxytryptamine is that it potentiates contractile responses to other vasoconstrictors such as angiotensin II (AngII), norepinephrine, prostaglandins, and histamine (Vanhoutte *et al.*, 1984). 5-hydroxytryptamine-induced contractions in isolated arteries are augmented by hypoxia (Vanhoutte *et al.*, 1984; Van Nueten, 1985) hypertension (Watts, 2002), and diet-induced obesity (Boustany-Kari *et al.*, 2007). An intact endothelium can protect from 5-hydroxytryptamine-induced contractions either by relaxant agonist or by degrading 5-hydroxytryptamine by endothelial monoamineoxidase. When the endothelium is damaged, vasoconstrictor responses to 5-hydroxytryptamine can be enhanced (Van Nueten, 1985).

Phenylephrine is a synthetic drug structurally similar to epinephrine (Aschenbrenner & Venable, 2009) and a well-known vasoconstrictor (Chen *et al.*, 1994) by acting through alpha$_1$(α_1)-adrenergic receptors in blood vessels, mainly located on vascular smooth muscle cells (Langer & Schoemaker, 1989; Piascik & Perez, 2001). Adrenergic receptors belong to the family of G-protein coupled receptors. Several different receptor subtypes exist: α_1, α_2, β_1, β_2, β_3. (Piascik & Perez, 2001) α_1 and α_2 adrenergic receptors mainly regulate vascular contraction (Piascik *et al.*, 1996). Phenylephrine causes vascular contraction by binding to the G-protein coupled α_1-adrenergic receptor. Activation of this receptor leads to stimulation of PLC which is involved in the production of IP_3 and DAG from PIP_2. Then, IP_3 increases intracellular Ca^{2+} by activation of Ca^{2+} channels and protein kinase C (PKC), leading to vasoconstriction (**Figure 1-8**) (Hein & Kobilka, 1995; Piascik *et al.*, 1996; Guimaraes & Moura, 2001).

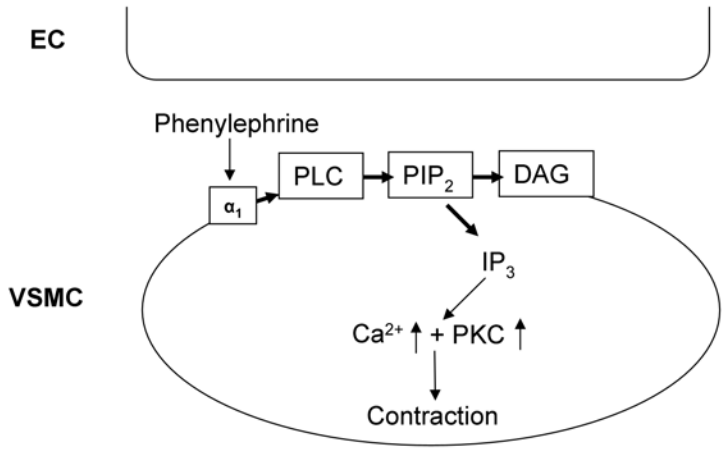

Figure 1-8 Phenylephrine-mediated contraction of vascular smooth muscle cells
Phenylephrine binds to α_1 adrenergic receptors on VSMCs activating PLC to produce IP_3 and DAG from PIP_2. IP_3 activates the release of intracellular Ca^{2+} and activates PKC which causes vasoconstriction of VSMCs. EC, endothelial cell; VSMC, vascular smooth muscle cell; α_1, α_1 adrenergic receptor; PIP_2 phosphatidylinositol 4, 5-bisphosphate; PLC, phospholipase C; IP_3, inositol 1, 4, 5-triphosphate; DAG, diacylglycerol; PKC, protein kinase C (Ponnuchamy & Khalil, 2009).

1.4 Obesity, oxidative stress, and vascular dysfunction

ECs produce low amounts of reactive oxygen species (ROS) under normal conditions but these amounts increase under pathological conditions such as obesity (Traupe *et al.*, 2002; Taniyama & Griendling, 2003; Faraci & Didion, 2004; Mundy *et al.*, 2007b). Accumulation of fat increases oxidative stress in humans and mice (Urakawa *et al.*, 2003) by up-regulation of NADPH oxidase and downregulation of antioxidant enzymes (Furukawa *et al.*, 2004). Most important ROS in the vasculature are superoxide anion (O_2^-), H_2O_2, and hydroxyl radical (•OH). O_2^- is generated by several different enzymes such as NADPH oxidase, xanthine oxidase, and also eNOS. Enhanced oxidative stress decreases NO bioavailability (Schulz *et al.*, 2008). Under certain conditions, mainly during pathophysiological conditions, eNOS produces O_2^- and NO when it is uncoupled which happens, if either L-arginine or the important cofactor BH_4 is not available sufficiently (Kojda & Harrison, 1999). O_2^- can scavenge NO leading to peroxynitrite ($ONOO^-$) which further lowers NO availability and thereby reducing endothelium-dependent relaxation and favoring endothelium-dependent contractions (Vanhoutte *et al.*, 2009). Expression of the important inflammatory cytokine TNFα is increased by high-fat diet and has been associated with obesity-related endothelial dysfunction by stimulating the production of O_2^- thereby reducing NO availability (Zhang *et al.*, 2009). However, O_2^- can also be dismutated by the antioxidant enzyme superoxide dismutase (SOD) to H_2O_2 which acts as an EDHF causing endothelium-dependent relaxation

(Shimokawa, 2010). On the other hand, H_2O_2 can be further converted by catalase to H_2O and O_2 or to •OH by the Fenton reaction (Dikalova *et al.*, 2001) or Haber-Weiss reaction (Ingelman-Sundberg & Johansson, 1984; Taniyama & Griendling, 2003; Kyaw *et al.*, 2004). Interestingly, •OH enhanced endothelium-dependent relaxation in mice fed a high-fat diet (Mundy *et al.*, 2007b). Furthermore, in mice high-fat diet feeding leads to an increase in endothelium-dependent contractions due to ROS, insulin resistance and increased expression of TP receptors (Gollasch, 2002; Traupe *et al.*, 2002; Mundy *et al.*, 2007b).

1.5 Mouse models of obesity

Nutrition rich in fat causes obesity in humans and is correlating with an elevation in body fat resulting in increased body fat mass (Klesges *et al.*, 1992; Tucker & Kano, 1992). Animal experiments with monkeys, pigs, dogs, hamsters, squirrels, rats, and mice have shown that diets rich in fat content cause obesity, too (West & York, 1998). First studies, in which rodents were fed with a high-fat diet, are based on rat studies in which rats were fed with a so called "cafeteria" diet (Rothwell & Stock, 1982). Rats were exposed to food which is eaten by humans such as chocolate, biscuits, bread, ham, cheese etc. One disadvantage of the "cafeteria" diet is that the quantification and analysis of diet intake is difficult to determine. Therefore, rodent-suitable, pelleted high-fat diets were introduced. It is important to note that high-fat diet feeding in animals not necessarily results in obesity. It is evident that some strains or species within one family are prone to become obese and some others not. In squirrel monkeys, high-fat diet feeding results in obesity whereas cebus monkeys do not become obese upon feeding with high-fat diet (Ausman *et al.*, 1981). This phenomenon is also observed in some dog, hamster, and mice species and strains (West *et al.*, 1992). West *et al.* investigated in nine inbred mouse strains which of these strains is suitable to study diet-induced obesity. Their results showed that the AKR/J mouse strain is the most obesity-sensitive strain whereas the SWR/J is rather resistant to diet-induced obesity (DIO) (West *et al.*, 1992). Other studies by York and colleagues demonstrated that the C57BL/6J mouse strain is prone to develop obesity during high-fat diet whereas the CAST/Ei strain is not (York *et al.*, 1996). These researchers identified a locus on chromosome 15 which is probably responsible to regulate fat content in the body (York *et al.*, 1996). These findings could explain that chromosomal variations play a role and that some mouse strains are more sensitive for the development of obesity than others. Interestingly, Rabot *et al.* showed that germ-free C57BL/6J mice are resistant to DIO and insulin resistance when fed with a high-fat diet compared to conventional mice (Rabot *et al.*, 2010). Nevertheless, the C57BL/6J mouse strain is a well tolerated model to study DIO because it develops severe obesity, hyperglycemia, hyperinsulinemia, and hypertension when fed with a high-fat diet (Surwit *et al.*, 1988), even though mice in general have high HDL levels and low LDL levels in their plasma compared to humans (Fox *et al.*,

2006) in which obesity can lead to the development of hypercholesterolemia (high LDL levels and low HDL levels) (Tershakovec *et al.*, 2002). Additionally, this strain is of particular interest because it develops metabolic changes similar to humans (Collins *et al.*, 2004). On the other hand, this strain remains physically and metabolically normal when held on a low-fat diet (Surwit *et al.*, 1988). Several different mouse models despite DIO mouse models also known as environmentally-induced obesity mouse models (Surwit *et al.*, 1988) exist to study obesity (Brownlow *et al.*, 1996). These are models in which spontaneous mutations occur leading to an obese phenotype (Chua *et al.*, 1996; Tschop & Heiman, 2001), transgenic mouse models in which a specific gene is overexpressed resulting in obesity (Shepherd *et al.*, 1993; Ludwig *et al.*, 2001), mouse models of gene disruption (Huszar *et al.*, 1997) and models in which a chemical or surgical manipulation is carried out (Tokunaga *et al.*, 1989). Most important mouse models of each different type are summarized in **Table 1-1**. Important to note is that weight loss in humans is caused by diets low in fat (Schaefer *et al.*, 1995) which is associated with many metabolic improvements. In animals, reduced dietary fat content can reverse obesity (Bartness *et al.*, 1992; Bell *et al.*, 1995), but also evidence exists that obesity is not reversed when animals are fed with a low-fat diet (Hill *et al.*, 1989). Interestingly, in C57BL/6J mice fed for 4 months with a high-fat diet followed by 4 months of a low-fat diet obesity and diabetes were reversible (Parekh *et al.*, 1998). Based on these findings, C57BL/6J mice are ideal to study high-fat diet-induced obesity as well as low-fat diet-induced weight loss. Even though the mentioned mouse models in **Table 1-1** are ideal to study DIO, extrapolation of the results from DIO studies to obesity in humans has to be regarded with caution. There are huge differences in body size, food intake, metabolic rates and body fat distribution between animals and humans (Hartung, 2008). Another factor to be included is that food which animals consume in nature is usually not composed of high fat (West & York, 1998). Other limitations are too small animal group sizes for statistical analysis, lethality (especially in transgenic mouse models) or exposure and durations of dietary treatments which do not reflect realistic human tolerance levels (Hartung, 2008).

Obesity model	Model description	Diet	Physiological outcome	Applicability of obesity mouse models to humans
C57BL/6J	Environmentally or diet-induced obesity by high-fat diet feeding	High-fat diet	Obesity, hyperglycemia, hyperinsulinemia, hypertension, insulin resistance	Humans develop obesity and diabetes upon nutrition high in fat
Lep^{ob}/ Lep^{ob}	Spontaneous mutation in the *leptin* gene→ termination of leptin synthesis	Normal diet	Early- onset morbid obesity, hyperphagia, hypothermia, hyperglycemia, hyperinsulinemia, reduced energy expenditure	Spontaneous mutation of the *leptin* gene is rare in humans
$Lepr^{db}$/ $Lepr^{db}$	Spontaneous mutation in the leptin receptor	Normal diet	Morbid obesity, hyperphagia, hyperleptinemia, insulin resistance, type 2 diabetes	Spontaneous mutation in the leptin receptor gene is rare in humans
GLUT-4 overexpression	Transgenic overexpression of GLUT-4 in adipose tissue	Normal diet	Early-onset obesity, greater basal and insulin-stimulated glucose transport in adipocytes, increase in number of fat cells	Overexpression of GLUT-4 not found in humans
MCH overexpression	Transgenic overexpression of *MCH* gene	High-fat diet	Late-onset obesity, type 2 diabetes	-
MC4-R deficiency	Gene disruption of MC4-R	Normal diet	Early-onset obesity, hyperphagia, hyperglycemia, hyperinsulinemia	Mutation in human MC4-R identified→ obesity, hyperphagia, hyperglycemia, hyperinsulinemia
PVN-lesion	Chemical or surgical-induced lesion of the PVN	Normal diet	Obesity, hyperphagia, hyperinsulinemia	-

Table 1-1 Common mouse models of obesity
Lep^{ob}/Lep^{ob}, spontaneous mutation in the *leptin* gene; $Lepr^{db}$/$Lepr^{db}$, spontaneous mutation in the leptin receptor; GLUT-4, glucose transporter type-4; MHC, melanin concentrating hormone; MC4-R, melanocortin-4 receptor; PVN, paraventricular nucleus.

1.6 Cytochrome P450 enzymes

1.6.1 Cytochrome P450 enzymes: general overview

The cytochrome P450 (Cyp450) enzymes belong to a superfamily of proteins containing heme, acting as monooxygenases catalyzing the oxidation of endogenous substrates (e.g. steroids such as testosterone and progesterone or fatty acids such as arachidonic acid) and exogenous compounds (e.g. xenobiotics such as drugs (acetaminophen), toxins (ethanol, carbon tetrachloride) and pro-carcinogens (nitrosamines) (Guengerich, 1987; Niwa *et al.*, 2009).

The monooxygense reaction is following:

$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O$

It means that a substrate (RH) binds to the heme iron in ferric state (Fe^{3+}). This promotes the reduction of the heme group from a ferric state to a ferrous state (Fe^{2+}) by an electron transfer from cytochrome P450 reductases. This ferrous hemoprotein can now bind O_2 to form an oxycomplex which is further reduced by a second electron

transfer to a peroxycomplex. Protonation of the peroxycomplex leads to the cleavage of O-O bond producing H_2O and the oxenoid complex which inserts the other oxygen atom into the substrate RH to result in the product (ROH) (Lewis & Pratt, 1998; Davydov, 2001). ROS generation will occur during P450 catalytic cycle if transfer of the electrons is used for the reduction of oxygen to O_2^- instead of Fe^{2+}or if the

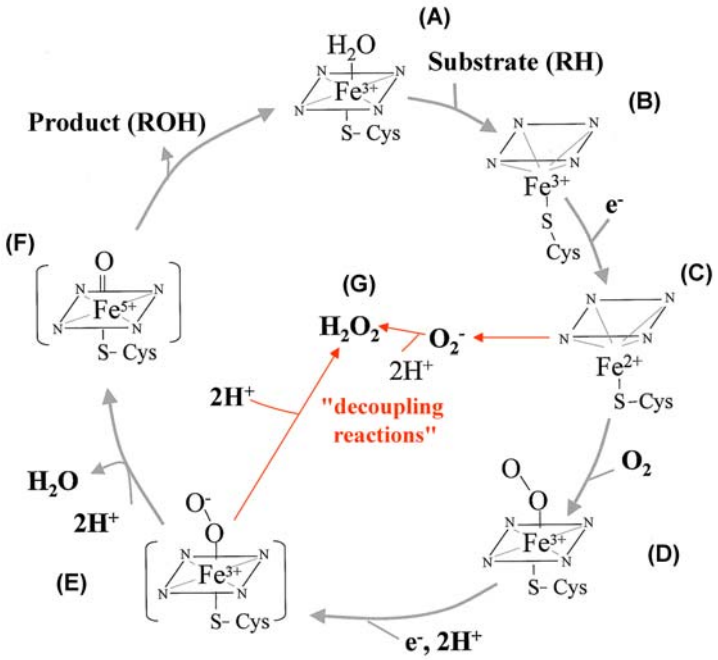

Figure 1-9 Catalytic cycle of cytochrome P450 enzymes
The substrate (RH) binds to the active site of Cyp450 enzymes containing a heme iron in ferric state (Fe^{3+}) with H_2O at one coordination position and a cystein residue at the other coordination position **(A)**. Binding of the substrate RH leads to a conformational change of the active site displacing H_2O and promoting the reduction of the heme group from a Fe^{3+} **(B)** to a ferrous state (Fe^{2+}) by an electron transfer from NAD(P)H via NADPH-cytochrome P450 reductase or other reductases **(C)**. O_2 can now bind to heme iron of the intermediate to form an oxycomplex **(D)** which is further reduced by a second electron transfer to a negatively charged peroxycomplex **(E)**. Then, this peroxycomplex is immediately protonated ($2H^+$) leading to the cleavage of O-O bond thereby releasing one oxygen atom in form of H_2O and producing the oxenoid complex **(F)** which inserts the other oxygen atom into RH to result in the product (ROH). ROS generation will occur during P450 catalytic cycle if transfer of the electrons is used for the reduction of oxygen to O_2^- instead of Fe^{2+}or if the peroxycomplex is protonated resulting in H_2O_2 (reactions indicated by red arrows) This is known as the "decoupling reaction" **(G)**. Adapted from (Fleming, 2001).

peroxycomplex is protonated resulting in H_2O_2. This is known as the "decoupling reaction" (**Figure 1-9**) (Loida & Sligar, 1993). Binding of CO to reduced Cyp450 enzymes inhibits the catalytic cycle (Fabry & Lieber, 1979). Irradiation with light at a wavelength of 450 nm abolishes CO inhibition (Diehl *et al.*, 1969). The number 450 visible in the enzymes` name represents at which wavelength Cyp450 enzymes absorb light. Cyp450 enzymes are present in animals, plants, fungi, bacteria and archea (Danielson, 2002). A nomenclature system for eukaryotic and prokaryotic Cyp450 enzymes based on sequence homologies exist which is updated since 1995 (Nelson *et*

al., 1996; Nelson, 2009). The nomenclature is as follows: Cyp stands for cytochrome P450, followed by an arabic number which represents the family, the capital letter indicates the subfamily and the last Arabic number describes the individual gene within the subfamily. The number of Cyp450 genes varies between humans and mice. In humans, 57 Cyp450 genes with annotated function and 58 pseudogenes are known, whereas mice have 102 functional Cyp450 genes and 88 pseudogenes (Nelson *et al.*, 2004). The mammalian expression of Cyp450 enzymes is diverse and is reported in several different tissues including liver, kidney, adrenal, lung, gonads, brain, and cardiovascular tissue (Elbekai & El-Kadi, 2006; Seliskar & Rozman, 2007). All Cyp450 enzymes in mammals are membrane bound either to the endoplasmic reticulum (ER) membrane (representing 90 %) or the inner mitochondrial membrane (Bar-Nun *et al.*, 1980; Nelson *et al.*, 2004; Seliskar & Rozman, 2007).

1.6.2 Cytochrome P450 enzymes in the regulation of vasomotion

Many Cyp450 enzymes as well as their metabolites are implicated to regulate cardiovascular function and diseases (Fleming, 2001; Roman, 2002). Several different Cyp families including Cyp1, Cyp2, Cyp3, Cyp4, Cyp8, Cyp11 and Cyp19 were found to be expressed in cardiovascular tissue (Elbekai & El-Kadi, 2006). Cyp450 enzymes belonging to the Cyp2 family are found to be expressed in vascular endothelial cells (e.g. Cyp2B, Cyp2C, Cyp2J) (Roman, 2002; Elbekai & El-Kadi, 2006) whereas Cyp2E1 was identified in heart and coronary vessels in humans (Minamiyama *et al.*, 1999), but also in aorta (Irizar & Ioannides, 1995; Elbekai & El-Kadi, 2006). The most important endogenous substrate of Cyp450 enzymes is the unsaturated fatty acid AA which is metabolized either to epoxyeicosatetraenoic acids (EETs) and dihydroxyeicosatetraenoic acids in liver but in vascular tissue predominantly EETs and hydroxyeicosatetraenoic acids (HETEs) are formed (Roman, 2002). AA can also be metabolized by COX to prostaglandins or by LOX to leukotrienes known to have vasoactive functions. **Figure 1-10** summarizes the metabolism of AA. EETs are important vasodilators generated by epoxygenases such as by Cyp2B, Cyp2C, Cyp2J. They are also known as EDHFs because vasodilator responses by EETs are mediated through NO and PGI_2-independent vasodilatation by the activation of large conductance Ca^{2+}-activated K^+ channels (BK_{ca}) on VSMCs leading to hyperpolarization and relaxation (Michaelis & Fleming, 2006). An important HETE, known to act as a vasoconstrictor, is 20-HETE which is metabolized from AA by enzymes of the Cyp4 family, mainly the ω- hydroxylase Cyp4A (Fleming, 2001). 20-HETE-mediated vasoconstriction can be initiated by stretch of the membrane or in response to AngII or norepinephrine (Uddin *et al.*, 1998; Muthalif *et al.*, 2000; Roman, 2002). This leads to the activation of PLC releasing IP_3 and DAG. IP_3 activates the release of intracellular Ca^{2+} which leads to stimulation of PLA_2 and DAG lipase releasing AA from membrane PLs in VSMCs and consequently to the generation of 20-HETE. Then, 20-HETE inhibits BK_{ca} in VSMCs leading to depolarization of VSMCs which activates influx of

Ca^{2+} by L-type, voltage sensitive Ca^{2+} channels resulting in vasoconstriction (Roman, 2002).

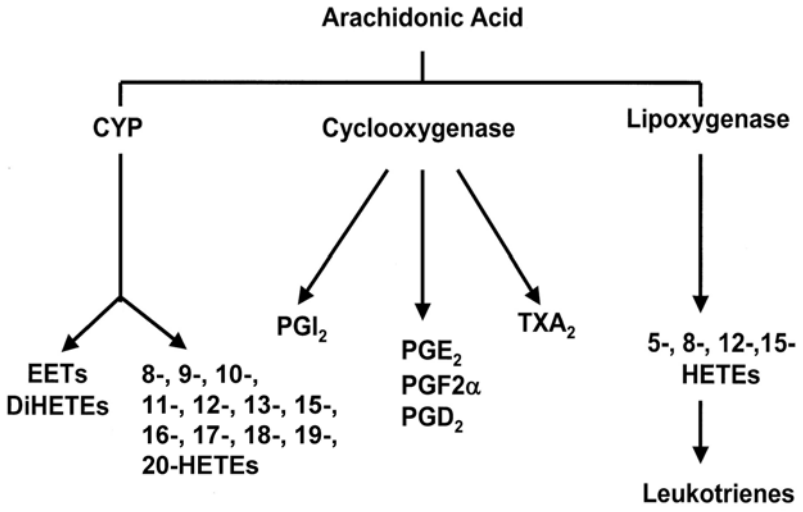

Figure 1-10 Metabolism of arachidonic acid
The metabolism of arachidonic acid (AA) includes three different enzyme classes such as Cytochrome P (Cyp) 450 enzymes, cyclooxygenase and lipooxygenase. Several different vasoactive metabolites are formed. Epoxyeicosattrienoic acids (EETs), dihydroxyeicosatetraenoic acids (DiHETEs), hydroxyeicosatetraenoic acids (HETEs), Prostacylin (PGI_2), prostaglandin E_2 (PGE_2), prostaglandin $F2_\alpha$ ($PGF2_\alpha$), prostaglandin D_2 (PGD_2), thromboxane A_2 (TXA_2) (Roman, 2002).

1.6.3 Cyp2E1 in liver

Cyp2E1 plays a pivotal role in ethanol oxidation in liver and liver injury (Cederbaum *et al.*, 2009). Many years it was believed that alcohol dehydrogenase alone catalyzes the conversion of ethanol to acetaldehyde *in vivo* (Lieber, 1992). *In vitro*, catalase can convert ethanol to acetaldehyde via an H_2O_2 generating system (Crabb & Liangpunsakul, 2007). Lieber and collegues demonstrated that in chronic ethanol consumption ethanol is metabolized by the microsomal ethanol-oxidizing system. Cyp2E1 was found to be the regulatory enzyme oxidizing ethanol to acetaldehyde (Lieber, 1999; 2004). Chronic ethanol consumption increases Cyp2E1 levels, ROS generation, and lipid peroxidation in the liver (Lu & Cederbaum, 2008). It can produce ROS during the catalytic cycle which plays a role in liver injury (Cederbaum *et al.*, 2009). It is mainly expressed in liver, specifically in the centrilobular zone (also known as zone 3) of liver lobules (Ingelman-Sundberg *et al.*, 1988). Liver hepatocytes and Kupffer cells also express Cyp2E1, and its expression is inducible by ethanol (Koivisto *et al.*, 1996; Lieber, 1997) but also by pyrazole, 4-methylpyrazole, acetone (Jimenez-Lopez & Cederbaum, 2005). Pathophysiological conditions such as obesity (Dey & Cederbaum, 2007), diabetes (Schafer *et al.*, 2010), and starvation (Lieber, 1997) can influence its expression. Cyp2E1 expression is regulated on transcriptional, posttranscriptional, and posttranslational levels (Lieber, 1997). In obese (Salazar *et al.*,

1988; Raucy *et al.*, 1991) and diabetic rats (Sindhu *et al.*, 2006) Cyp2E1 expression in liver is increased. Interestingly, in Lep^{ob}/Lep^{ob} mice hepatic Cyp2E1 expression is reduced compared to control mice, (Enriquez *et al.*, 1999; Leclercq *et al.*, 2000; Sindhu *et al.*, 2006) indicating that leptin regulates the expression of Cyp2E1. As described above, most of Cyp450 enzymes are located in the membrane of the ER, which is also the case for Cyp2E1. It is tethered with its hydrophobic NH_2-terminus to the membrane while the C-terminus with the catalytic active site is directed to the cytosol (Jimenez-Lopez & Cederbaum, 2005). Interestingly, lack of Cyp2E1-containing hydrophobic NH_2-terminus leads to a transport of Cyp2E1 to the mitochondria (Neve & Ingelman-Sundberg, 1999; 2001). Additionally, a phosphorylated form of Cyp2E1 at serine129 in mitochondria is described which is inducible by pyrazole (Robin *et al.*, 2001). Cyp2E1 catalyzes the oxidation of several different endogenous and exogenous substrates. Important endogenous substrates are acetone and fatty acids such as AA. Acetone is metabolized by Cyp2E1 to acetol and methylglyoxal (Bondoc *et al.*, 1999). Exogenous substrates of Cyp2E1 are various including aromatic compounds (acetaminophen also known as paracetamol, chlorzoxazone, p-nitrophenol), alcohols (ethanol, methanol, propanol) nitrosamines (N-N Dimethylnitrosamine, N-N Diethylnitrosamine), halogenated alkanes and alkenes (chloroform, halothane, ethane, hexane), and reducible substrates (carbon tetrachloride) (Koop, 1992; Tanaka *et al.*, 2000). Conversion of some exogenous substrates by Cyp2E1 can cause toxic products such as acetaminophen which in combination with ethanol can cause hepatoxicity (Lu & Cederbaum, 2008). Enzyme activity of Cyp2E1 can be determined by hydroxylation of chlorzoxazone to 6-hydroxychlorzoxazone (Peter *et al.*, 1990) or of *p*-nitrophenol to 4-nitrocatechol (Chang *et al.*, 2006). A concentration of 200 µM of dietyldithiocarbamate (DETC) decreases hydroxylation reaction of aniline, a substrate of Cyp2E1, to aniline 4-hydroxylation. This effect is even more prominent at a concentration of 500 µM (Ono *et al.*, 1996). Guengerich and colleagues showed that chlorzoxazone hydroxylation which is catalyzed by Cyp2E1 is inhibited by DETC about 80-85% indicative of a reduced Cyp2E1 activity (Guengerich *et al.*, 1991).

1.6.3.1 Cyp2E1-deficient mice

Many Cyp450 enzymes play a very important role in metabolizing and activating various xenobiotics to products which are hepatotoxic. Cyp2E1 metabolizes acetaminophen (a worldwide known and clinically used analgesic and antipyretic) to *N*-acetyl-*p*-benzoquinone amine (NAPQI) (Corcoran *et al.*, 1980) which is usually inactivated by reacting with glutathione in the liver when low amounts are available. In case of a high dose of acetaminophen, NAPQI levels are increased and glutathione levels are decreased which may lead to an inhibition of NAPQI break-down by the liver. High amounts of NAPQI can act as free radicals destroying hepatocytes leading to liver damage and toxicity (Dahlin *et al.*, 1984; Lee *et al.*, 1996). To investigate whether Cyp2E1 plays a role in the hepatotoxicity of acetaminophen *in vivo*, Cyp2E1-deficient

(Cyp2E1$^{-/-}$) mice were generated by Lee *et al*. by knockout of Cyp2E1 through homologous recombination in embryonic stem cells (Lee *et al.*, 1996). Cyp2E1$^{-/-}$ mice are viable and phenotypically not different from wild type animals. Treatment of the mice with 400 mg of acetaminophen per kg ofbody weight, Cyp2E1$^{-/-}$ mice were resistant to this dose whereas 50 % of their wild type counterparts died. Very high doses of acetaminophen (up to 800 mg/kg body weight) were also lethal to Cyp2E1$^{-/-}$ mice. Additionally, levels of the liver enzymes aspartate aminotransferase and alanine aminotransferase were increased upon stimulation with acetaminophen in wild type mice but not in Cyp2E1$^{-/-}$ mice. An increase in those enzymes indicates hepatocyte cell death and gives an implication of how damaged the liver is. These results indicate that Cyp2E1 is involved in acetaminophen-induced toxicity in the liver (Lee *et al.*, 1996). Until now most researchers have utilized Cyp2E1$^{-/-}$ mice in investigating the role of Cyp2E1 in liver toxicity extensively, but no data is available with these mice to investigate the role of Cyp2E1 in vascular reactivity.

1.6.4 Cyp2E1 in the vasculature

Its role in liver is extensively investigated (Lieber, 1997), but little is known about the role of Cyp2E1 in the regulation of vascular reactivity. Cyp2E1 acts as a ω-hydroxylase catalyzing the formation of 18 (*R*)-, 19 (*R*)-and 19(*S*)-HETEs from AA in the vasculature, an endogenous substrate of Cyp2E1 (Laethem *et al.*, 1993). These metabolites play an important role in maintaining vascular homeostasis (Elbekai & El-Kadi, 2006). Zhang *et al*. showed that Cyp2E1 expression is down regulated in interlobar arteries of spontaneously hypertensive rats (SHR) compared to control Wistar Kyoto rats (WYR) (Zhang *et al.*, 2005). These researchers showed that 18- and 19-HETE levels are decreased in arteries of both rat groups by inhibition of Cyp2E1 with 500 μM DETC. This inhibitor is reported to inhibit Cyp2E1 in rat (Brady *et al.*, 1991) and human liver microsomes (Guengerich *et al.*, 1991), but also SOD (Ghanam *et al.*, 1998; Karasu, 2000), and Cyp6A (Ono *et al.*, 1996). The decrease in Cyp2E1-derived 18-and 19-HETEs leads to an amplification of Cyp4A-derived 20-HETE-mediated vasoconstriction and 20-HETE also enhances phenylephrine-mediated contractions (Zhang *et al.*, 2005), indicating that 18-and 19 HETE act as vasodilators. Another study by Escalante and coworkers showed that 20-HETE strongly contracts isolated aorta of rats, but also 19-HETE is able to contract this artery (Escalante *et al.*, 1989). COX inhibition by indomethacin and addition of a TXA_2 receptor antagonist SQ29458 abolished 20-HETE-mediated contractions, suggesting that 20-HETE is converted by COX to TXA_2 leading to the observed contraction (Escalante *et al.*, 1989).

1.7 Aim of the study

The prevalence of obesity has risen worldwide. Main reason responsible for the development of obesity is intake of nutrition rich in fat. As a consequence body weight is increased and is frequently associated with glucose intolerance and vascular dysfunction. Recent studies suggest that obesity is associated with an increased risk of cardiovascular diseases even after reduction of body weight. Little is known which genes play a role in obesity-associated vascular dysfunction even after reduction of body weight. To this end C57BL/6J mice were either fed with a control diet or a high-fat diet for 30 weeks or 15 weeks with a high-fat diet followed by 15 weeks of control diet.

Main objectives of my dissertation were:

(1) To investigate the impact of high-dietary fat content and its reduction on body weight, glucose tolerance, and vascular reactivity in C57BL/6J mice

(2) To investigate whether in aorta of these mice groups changes in steady state mRNA expression levels of genes (referred to as gene expression levels) and gene splicing occur

(3) To identify and validate target genes revealing changes in gene expression that sustain after high-dietary fat content is reduced

(4) To investigate whether the selected target gene Cyp2E1 is involved in the regulation of vascular reactivity in thoracic aorta of mice and characterize aortic Cyp2E1 splice variants

2 Materials and Methods

2.1 Materials

All chemicals were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany) if not it is otherwise stated. Oligonucleotide primers were obtained from Microsynth AG (Balgach, Switzerland), and described in Appendix **Table 5-1** and **Table 5-2**.

2.2 Methods

2.2.1 Mice and dietary treatment

Mice were housed at the animal facility of the University Hospital of Zurich (Biologisches Zentrallabor). Animals had access to tap water *ad libitum* and were kept in a 12h: 12h light-dark cycle. Housing facilities and mice experiments were approved by the local authorities for animal research (Kommission für Tierversuche des Kantons Zürich, Switzerland) and conform to the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

An established DIO mouse model was used (West *et al.*, 1992): Young (4 weeks old), male C57BL/6J (Charles River, Sulzfeld, Germany) mice were assigned to one of the 3 dietary protocols for 30 weeks: Control diet (CD) group was fed with standard chow for 30 weeks (12.3 % kcal from fat, Kliba Nafag 3430, Kaiseraugst, Switzerland), mice included in the high-fat diet (HFD) group were fed with a high-fat diet (41% kcal from fat, Research Diets D12079B, New Brunswick, NJ), for 30 weeks, and the mice in the high-fat diet/control diet (HFD/CD) group were fed with a high-fat diet (41% kcal from fat, Research Diets D12079B, New Brunswick, NJ) for 15 weeks followed by 15 weeks of control diet (12.3 % kcal from fat, Kliba Nafag 3430, Kaiseraugst, Switzerland) (**Table 2-1**).

Diet constituents	CD	HFD
Fat	12.3 %	41 %
Carbohydrate	65.4 %	45 %
Protein	22.4 %	17 %

Table 2-1 Main nutritional constituents in CD and HFD
Values represent percent of kcal from fat, carbohydrate and protein.

Breeding pairs of Cyp2E1-deficient (129/Sv-*Cyp2e1*^{tm1Gonz}/J) mice and corresponding wild type (129S1/SVImJ) mice were obtained from Prof. Frank J. Gonzalez at the National Cancer Institute, Bethesda, Maryland, USA. *Cyp2E1* gene was disrupted by homologous recombination in embryonic stem cells. Exon 2 of *Cyp2E1* was replaced with a neomycin resistance cassette (Lee *et al.*, 1996). Mice were genotyped by PCR using isolated genomic DNA (deoxyribonucleic acid) from wild type and Cyp2E1-deficient mice. For genotyping wild type forward (5`-AGTGTT CACACTGCACCTGG-3`) and reverse (5`-CCTGGAACACAGGAATGTCC-3`) primer and corresponding mutant forward (5`-CTTGGGTGGAGAGGCTATTC-3`) and reverse (5`-AGG TGAGATGACAGGAGATC-3`) primer were used published at the Jackson laboratory website to detect wild type and mutant PCR band at 125 and 280 base pairs (bp), respectively (The Jackson Laboratory.). Following PCR program was used for amplification on an iCycler™ Thermal Cycler PCR machine (BioRad Laboratories, Hercules, CA, USA):

Cycle (1x) Denaturation at 94°C for 3 min
Cycle (30x) Denaturation at 94°C for 20 seconds
Annealing at 58°C for 30 seconds
Elongation at 72°C for 35 seconds
Cycle (1x) Final elongation at 72 °C for 2 minutes

Amplified PCR products were separated on a 1.5 % agarose gel, visualized with UV light, photographed using the Molecular Imager® ChemiDoc™ XRS Imaging System (BioRad Laboratories, Hercules, CA, USA) and sequenced (Microsynth AG, Balgach, Switzerland).

Breeding of the animals was performed in the animal facilities at the Institute for Labortierkunde, University of Zurich. Male and female litters of wild type and Cyp2E1-deficient mice were fed with control diet (12.3 % kcal from fat, Kliba Nafag 3430, Kaiseraugst, Switzerland) for 8 weeks before vascular function experiments were performed.

2.2.2 Glucose tolerance test

Body weight of DIO animals was monitored weekly during the dietary treatment of 30 weeks. At the end of the feeding period glucose tolerance test (GTT) was performed with overnight fasted animals. For baseline glucose measurements (0 min) venous blood was taken from the tail vein. Then mice were injected with 2 mg/g of body weight D-glucose (Riedel-de Haën, Seelze, Germany), and blood was collected at 5, 10, 15, 30, 45, 60, 90, and 120 min. Glucose concentration in the blood was determined using an AccuChek Advantage glucose meter (Roche Diagnostics, Switzerland).

2.2.3 Vascular function experiments

2.2.3.1 Experimental set-up of organ chamber baths

After 30 weeks of dietary treatment, fasted mice were anesthetized and exsanguinated via cardiac puncture. Organs were removed and immediately snap-frozen at -80° C. In the Appendix **Figure 5-1**, a schematic representation of the experimental set-up of organ chamber baths used for vascular function experiments is shown. The aorta was carefully excised and placed in 4°C Krebs Ringer bicarbonate buffer solution of pH 7.4 (Bhattacharya *et al.*, 2008). Ascending aorta, aortic arch, descending aorta and thoracic aorta sections were carefully cleaned of adherent, connective fat and muscle tissue. Care was taken while cleaning of adherent tissue not to damage the endothelial and vascular smooth muscle cell layer. Ascending aorta, aortic arch and descending aorta (in one piece) were immediately snap-frozen at -80°C and stored for RNA isolation (for DNA exon microarray experiments). Thoracic aorta was cut into 3 mm rings, mounted onto two tungsten wires (100 µm diameters) and transferred into pre-warmed water-jacketed organ baths containing warm (37°C) gassed (95 % O_2, 5% CO_2) Krebs Ringer bicarbonate buffer solution of pH 7.4 (Bhattacharya *et al.*, 2008). Rings were connected to a force transducer to record isometric tension. First, a passive tension was induced to the rings by stretching the vessels step by step to an optimal weight of 3 g (Russell & Watts, 2000). After optimal passive tension was reached, rings were two times exposed to potassium chloride (KCl, 100 mM) until a stable contractile response was reached to test integrity of VSMC layer. Changes in vascular reactivity were recorded using a plotter (Rikadenki Elektronics GmbH, Freiburg, Germany).

2.2.3.2 Experimental protocols

The following protocols were used to determine vascular reactivity:

1. To investigate vascular contraction induced by phenylephrine and relaxation induced by acetylcholine in thoracic aorta from different dietary treatment groups, and wild type (WT) and Cyp2E1$^{-/-}$ mice, vessel rings were treated without and with DETC (100 µM or 500 µM) for 30 minutes before contraction with cumulative concentrations of phenylephrine (0.1 nM-100 nM). The contractions were induced to 60 % (in case of mice from dietary treatment groups) or 50 % (in case of WT and Cyp2E1$^{-/-}$ mice) of KCl-induced contraction. After a stable contractile plateau was reached increasing

concentrations of acetylcholine (0.1 nM-100 μM) were added to investigate endothelium-dependent relaxation (Appendix **Figure 5-2A**).

2. To investigate endothelium-dependent contractions and the effect of DETC on these contractions in thoracic aorta, vessels were incubated with the NO-synthase inhibitor N^G-nitro-L-arginine methylester (L-NAME, 300 μM) alone or with L-NAME and DETC (100 μM) for 30 minutes before pre-contracting the vessels with increasing concentrations of phenylephrine (0.1 nM-1 μM). Inhibition of NO using L-NAME is needed to elicit endothelium-dependent contractions at high concentrations of acetylcholine (>0.1μM) (Vanhoutte & Tang, 2008). After a stable contraction with phenylephrine was reached, acetylcholine in cumulative concentrations (0.1 nM-100 μM) (in case of mice from dietary treatment groups) or a single concentration (100 μM) (in case of WT and Cyp2E1$^{-/-}$ mice) was added (Appendix **Figure 5-2B**). To investigate whether endothelium-dependent contractions are mediated by COX, only vessels of Cyp2E1$^{-/-}$ mice were pre-incubated with L-NAME (300 μM) together with the non-selective COX inhibitor, meclofenamate (1 μM) (Kretz *et al.*, 2006) for 30 minutes prior to pre-contraction with phenylephrine (0.1 nM- 1 μM) followed by a single concentration of 100 μM of acetylcholine.

3. To investigate 5-hydroxytryptamine-mediated contractions in thoracic aorta from dietary treatment groups, and WT and Cyp2E1$^{-/-}$ mice, vessels were incubated without or with DETC (100 μM or 500 μM) for 30 minutes before rings were exposed to additive concentrations of 5-hydroxytryptamine in a concentration-dependent manner (0.1 nM-10 μM) (Appendix **Figure 5-2C**).

2.2.3.3 Statistical analysis of vascular function experiments

Statistical analyis was performed using the software Statview® for Windows® version 5.0.1. Two-tailed paired or unpaired Student`s t-test, one-way analysis of variance (ANOVA) for repeated measurements followed by Bonferroni correction were used, when appropriate. Data are represented as means ± SEM (standard error of the mean). n indicates the number of animals used in the experiments. A p value < 0.05 was considered significant.

2.2.4 DNA exon microarray and analysis

2.2.4.1 RNA extraction and array hybridization

5-10 mg aortic tissue originating from ascending aorta, aortic arch, and descending aorta/per mouse (3 replicates per treatment group were used) was disrupted by homogenization of the tissue using six borosilicate glass beads (3 mm) in the FastPrep® -24 System from MP Biomedicals (Solon, OH, USA). The FastPrep® -24 homogenizer uses a unique, vertical angular optimized motion to disrupt the tissue through a multidirectional, simultaneous beating to release RNA into the protective buffer very fast. Very fibrous tissue, such as aortic tissue, can be disrupted within seconds. The homogenization protocol for complete disruption of each aortic tissue/per mouse was

set as follows: 3 x 30 seconds at 6.5 m/s for followed by 1 x 30 seconds at 6.0 m/s. All other steps of RNA isolation were done as described in the RNeasy Fibrous Tissue Mini protocol from Qiagen AG (Hombrechtikon, Switzerland). Before cDNA synthesis, quality of the isolated RNA was determined using NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA). cDNA was synthesized from total RNA using a primer mix and reverse transcriptase using the WT (Whole Transcript)-Ovation™ Pico RNA Amplification System from NuGEN (Bemmel, the Netherlands) which is a rapid method to amplify cDNA from low amounts of total RNA. The primers have a DNA portion that hybridizes either to the 5`portion of the poly-A sequence or randomly across the transcript. SPIA® (Single Primer Isothermal Amplification), a linear isothermal DNA amplification process, was used to prepare single-stranded cDNA in antisense direction of the mRNA starting material. Single-stranded cDNA quality was determined using NanoDrop ND 1000 Spectrophotometer and Bioanalyzer 2100. Single stranded cDNA quality (3 µg) was converted into sense target-cDNA (ST-cDNA) using WT-Ovation™ Exon Module from NuGen. Fragmentation and biotin-labelling of ST-cDNA were generated with the FL-Ovation cDNA Biotin Module V2 from NuGEN. Biotin-labelled single-stranded cDNA targets (5µg) were mixed with a hybridization mix containing hybridization controls and the control oligonucleotide B2 from Affymetrix (Santa Clara, CA, USA). Samples were hybridized to Affymetrix Mouse Exon 1.0 ST Arrays (9 Exon Arrays were used because of 3 replicates per treatment group) for 18 h at 45 °C. Arrays were then washed using an Affymetrix GeneChip® Fluidics Station 450 FS450 0001 protocol. An Affymetrix GeneChip® Scanner 3000 was used to measure fluorescent intensity emitted by the labelled target. Transformation of Raw (Pixel) Images (DAT. file) of each array to signal intensity values (CEL. file) was performed using the Affymetrix® GeneChip® Command Console® (AGCC) software.

2.2.4.2 Statistical analysis of Microarray data

The microarray data provided exon-specific measurements of expressed genes in the 3 different experimental groups. Briefly, the Affymetrix Mouse Exon 1.0 ST Array contains ~ 4.5 million probes corresponding to ~ 1.2 million probesets which target ~ 1 million exons (Affymetrix Mouse Exon 1.0 ST Array). Each probe set consists of 4 probes which cover one exon of a particular gene (**Figure 2-1**).

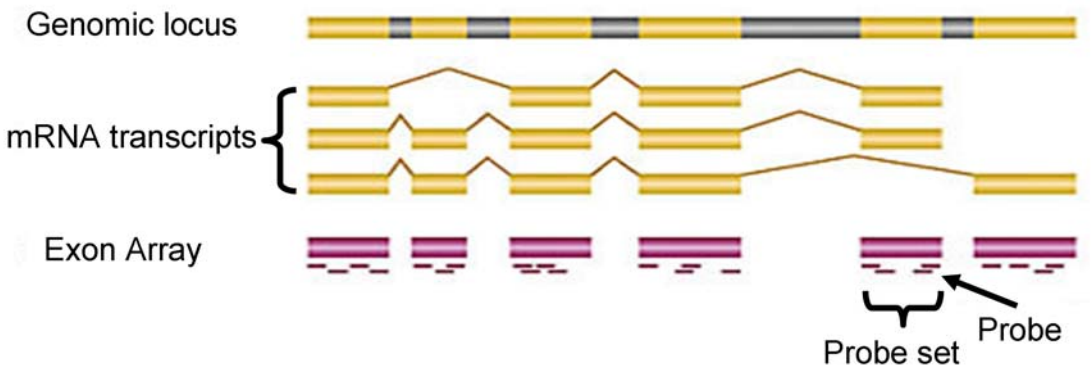

Figure 2-1 Schematic representation of mRNA transcripts originating from a genomic locus and design of the Affymetrix Mouse Exon 1.0 ST Array
A gene is a region of the genomic locus which can contain exons (yellow region) and introns (grey region). First, it is transcribed into pre-mRNA (precursor-messenger ribonucleic acid, still containing exons and introns). During Splicing, introns but also exons can be removed from the pre-mRNA leading to several different mRNA transcripts. This process is referred to as alternative splicing. In exon arrays 4 probes (pink dashes) cover one exon (pink region). 4 probes are one probe set which cover one exon of a particular gene. Adapted from Affymetrix data sheet: GeneChip®Exon Array System for Human, Mouse, and Rat (Affymetrix., 2005a).

In general, data analysis begins with pre-processing of the Exon array data. First, intensity values of each individual probe on each array chip are normalized to remove systematic errors (biases) from the data so that multiple chips can be compared to each other and analyzed together. Next step includes the generation of expression summaries by using the robust multi-array average algorithm in which intensity values of each individual probe in a probe set are summarized to a single intensity value (exon intensity value or so called probe set intensity value representing exon expression, **Figure 2-2**), and additionally, all probe set intensity values belonging to a gene are integrated to a meta-probe set intensity value (**Figure 2-2**) representing whole gene expression.

After pre-processing of the data, exonmap (Okoniewski *et al.*, 2007), a BioConductor/R package for Exon array analysis was applied to (i) identify genes that show a whole gene expressional change (meta-probe set expressional change representing gene expression), and to (ii) identify genes in which least 2 exons show an expressional change (probe set expressional change representing exon expression) in arterial tissue in any of the groups using ANOVA test.

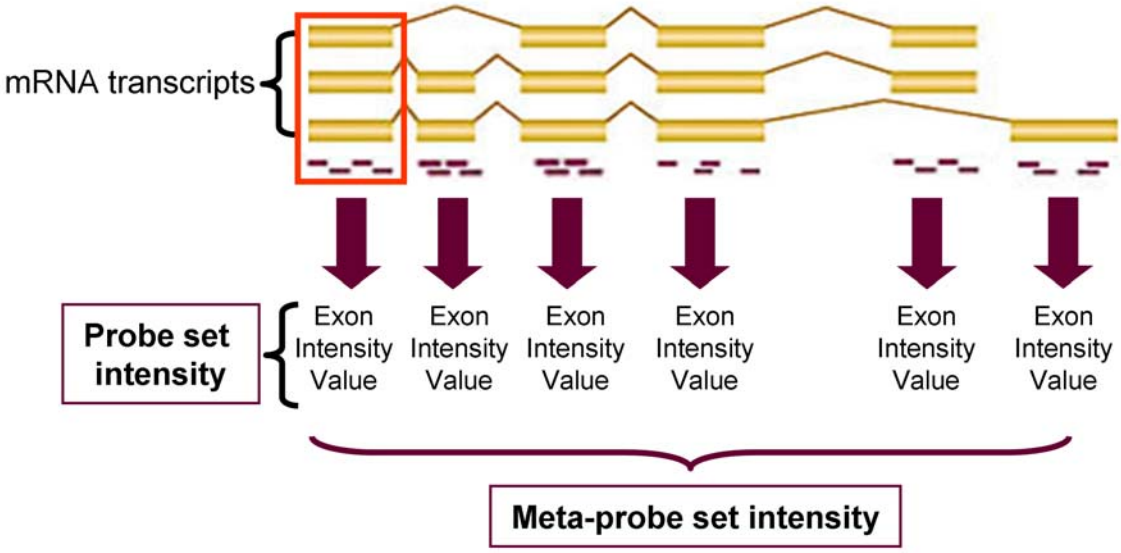

Figure 2-2 Summarization of probe set and meta-probe set intensities
Intensity values of each individual probe (pink dashes) in a probe set covering one particular exon are summarized to generate a single exon/probe set intensity value. As during splicing several different mRNA transcripts or isoforms of a particular gene can be generated, the summarized single exon intensity value is calculated based on the signals coming from one exon occurring in different mRNA transcripts of a particular gene (red box). Summarization of all exon/ probe set intensity values result in a meta-probe set intensity value representing whole gene expression. Adapted from Affymetrix data sheet: GeneChip®Exon Array System for Human, Mouse, and Rat (Affymetrix., 2005a).

(i) To find out genes that show a whole gene expressional change in any of the groups the analysis was done as follows:

Step 1: Differentially expressed probe sets/exons were identified by calculating the mean intensity value of each probe set/exon ($\log_2$ signal) of 3 replicates per treatment group.

Step 2: These significantly identified probe sets/exons were further mapped to the X:MAP Database (Yates *et al.*, 2008), a database of exon array annotation. Differentially expressed probe sets/exons can be mapped *in silico* to the entire genome to identify corresponding genes that show a whole gene expressional change in any of the groups. To find out those, the mean intensity value of each probe set/exon of 3 replicates per treatment group was summarized to a whole gene intensity value (meta-probe set intensity value). To generate a top list of genes showing a whole gene expressional change in any of the groups, only genes with a p value < 0.01 and a $\log_2$ effect (fold change) > 0.5 were included.

(ii) To generate the list of genes in which at least 2 exons show a significant expressional change in any of the groups the analysis was done as follows:

Step A: same as in Step1

Step B: Differentially expressed probe sets were mapped to the X: MAP Database to identify corresponding genes where at least 2 exons show a significant expressional change in any of the groups. In this case, the mean intensity values of each probe set/exon of 3 replicates per treatment group were not averaged to a whole gene intensity value. Here, only genes were included in the list where at least 2 exons demonstrated significantly different mean intensity values of each probe set/exon (probe set intensity value) of 3 replicates per treatment group. Genes were included with a p value threshold < 0.65 and a $\log_2$ effect (fold change) > 0.3.

The differences in intensity values between the treatment groups were visualized as line graphs using exonmap, by plotting each intensity value (y-axis, in $\log_2$ scale) against the total number of probe sets/exons of a particular gene (x-axis).

Gene Ontology analysis was performed using the pathway analysis software GeneGoMetacore (GeneGo, Inc.).

2.2.4.2.1 Tukey`s post-hoc test

To identify specific exons and corresponding genes that are significantly changed between HFD or HFD/CD and CD group Tukey`s post-hoc test was applied. Tukey`s post-hoc test was used based on the one-way ANOVA results. The list generated includes identified exons and corresponding genes with a p value < 0.05 and an exon fold change which is at least 0.5 up or down regulated compared to the CD group.

2.2.5 Quantitative real-time-polymerase chain reaction

Quantitative real-time-polymerase chain reaction (qRT-PCR) was performed using specifically designed mouse primers for the genes of interest (**Table 5-1**). Briefly, iQ SYBR Supermix PCR kit (1X final concentration; BioRad Laboratories, Hercules, CA, USA), primers (20 µM final concentration) and cDNA (1 ng/µl final concentration) were used to perform a two-step PCR with the following PCR program on the iCycler iQ™ real-time PCR detection system (BioRad Laboratories, Hercules, CA, USA):

Cycle (1x)	Denaturation at 95°C for 3 minutes	
Cycle (40x)	Denaturation at 95°C for 15 seconds	(step 1)
	Annealing and Elongation at 60°C for 1 minute	(step 2)

At the end of each extension step fluorescence was measured. Amplicons were confirmed by melting curve analysis, agarose gel electrophoresis and sequencing (Microsynth AG, Balgach, Switzerland). Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). The relative amount of each mRNA was normalized to the housekeeping gene β-actin and expressed as arbitrary units (AU).

2.2.6 "Exon-specific" PCR

Briefly, to perform PCR reaction RNA from liver and aorta of mice in the CD, HFD and HFD/CD group was reverse transcribed to cDNA and used at a final concentration of 1 ng/µl. Mouse specific exon-spanning primers (Appendix **Table 5-2**) for each exon of *Cyp2E1* were designed to perform PCR reactions. Following PCR program was used to amplify DNA fragments in an iCycler™ Thermal Cycler PCR machine (BioRad Laboratories, Hercules, CA, USA):

Cycle (1x)	Denaturation at 94°C for 5 min
Cycle (38x)	Denaturation at 94°C for 30 seconds
	Annealing at 60°C for 30 seconds
	Elongation at 72°C for 2 minutes
Cycle (1x)	Final elongation at 72 °C for 10 minutes

Amplified PCR products were separated on a 2 % agarose gel, visualized using UV light, photographed using the Molecular Imager® ChemiDoc™ XRS Imaging System (BioRad Laboratories, Hercules, CA, USA) and sequenced (Microsynth AG, Balgach, Switzerland)

2.2.7 Protein extraction and Western blot

Total protein was extracted from mouse aorta and liver using 100 µl radioimmunoprecipitation assay buffer (0.25 % Na-deoxycholate, 0.05 M Tris/HCl pH 7.4, 0.15 M NaCl, 0.001 M sodium ethylenediaminetetraacetic acid (Na-EDTA), 0.1% sodium dodecyl sulfate (SDS), 1 % NP-40) supplemented with a protease inhibitor cocktail, EDTA-free (1:7, Roche Diagnostics GmbH, Mannheim, Germany) at 4°C. Tissue homogenization was performed using TissueLyser (Qiagen AG, Hombrechtikon, Switzerland) 3 times at 30 Hz for 5 minutes. Finally, samples were centrifuged at 15700 rcf (13000 rpm) (Centrifuge type 5415R from Vadaux-Eppendorf AG, Basel, Switzerland) for 10 minutes at 4°C. Supernatants were collected and protein concentration was measured using the Bradford protein assay. As protein standard bovine serum albumin was used at concentrations 0, 7.81, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml (Kruger, 2002). Before using the samples in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 4X sample buffer (62.5 mM Tris pH 6.8, 10 % glycine, 2 % SDS, 0.01 % Bromphenol blue (3',3",5',5"-tetrabromophenolsulfonphthalein), 50 mM Dithiothreitol was added and incubated for 5 minutes at 95°C. Equal amounts of proteins (aorta: 25 µg, liver: 5 µg) and a molecular weight standard (SeeBlue®Plus2 Pre-Stained Standard, Invitrogen, Basel, Switzerland) were loaded and separated by SDS-PAGE using NuPage® 4-12 % bis-Tris gradient gels (Invitrogen AG, Basel, Switzerland) in 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (1X runnig buffer was used: 0.1 M MOPS, 0.1 M Tris-Base, 0.002 M Na-EDTA, 0.007 M SDS, 1l distilled H_2O) at 200 Volts for 50 minutes. Proteins were either blotted

onto a Polyvinylidene fluoride membrane (Millipore AG, Zug, Switzerland) after wetted in methanol or onto a Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences, part of GE Healthcare, Glattbrugg, Switzerland), depending on the protocol, using a wet tank electro blotting system (BioRad Laboratories, Hercules, California, USA) filled with 1X transfer buffer (25 mM Tris-Base, 0.19 M glycine, 3.47 mM SDS, 10 % methanol, 1l distilled H_2O) at 110 mV/per membrane for 2h. After transfer of the proteins, membranes were washed (phosphate buffered saline (PBS)-Tween 20 (0.1 %) or PBS-Tween 20 (0.2 %) or TBS-Tween 20 (0.1 %) 3 times 5-10 minutes before incubating the membrane in blocking buffer for 1h at room temperature on a shaker. Depending on the primary antibody, different dilutions and blocking buffers (**Table 2-2**) were used to incubate membranes overnight at 4°C on a shaker.

After over night incubation with one of the primary antibodies, blots were washed again as mentioned above and the appropriate horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse (GE Healthcare, Zurich, Switzerland) IgG secondary antibodies were added, diluted 1:5000 or 1:2000, respectively in blocking buffer for 1-1.5 h. Finally, membranes were washed as before and incubated for 5 minutes in

Primary antibody	Manufactureur	Dilution	Blocking buffer
Rabbit polyclonal antibody against full-length Cyp2E1	Abcam, Cambridge, UK	1:2000	1X gelatine blocking buffer: 0.15 M NaCl, 0.005 M EDTA pH 8, 0.05 M tris-HCl pH 7.5, 0.05% Triton X-100, 1l distilled H2O
Rabbit polyclonal antibody against a synthetic peptide corresponding to C-terminal Cyp2E1	Abnova, Taipei City, Taiwan	1:250	1X gelatine blocking buffer
Rabbit polyclonal antibody against N-terminal Car3	Abcam, Cambridge, UK	1:100	3% non-fat milk in PBS + 0.1% Tween 20
Mouse monoclonal antibody against Cidec	Abnova, Taipei City, Taiwan	1:500	5% non-fat milk in 1X TBS + 0.1% Tween 20
Rabbit polyclonal antibody against Erk1/2	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA	1:1000	1X gelatine blocking buffer

Table 2-2 Primary antibodies used for Western Blot

enhanced chemiluminescence (ECL)™ Western Blotting detection reagents (ECL, GE Healthcare, Zurich, Switzerland) before bands of interest were detected and visualized on high performance chemiluminescence films (Amersham, GE Healthcare, Zurich, Switzerland). Films were developed on the developing machine Cavomat 2000 IR (CAWO Photochemisches Werk GmbH, Schrobenhausen, Germany). Densitometry quantification of Western Blots were analyzed using Image J (Image Processing and Analysis in Java) software (developed by Wayne Rasband, National Institutes of Health

(NIH), Bethesda, MD, USA) and determined by dividing the absolute intensity of each sample band per group by the absolute intensity of the loading control band per group resulting in a relative intensity.

3 Results

3.1 Impact of diets on appearance, body weight and glucose tolerance in C57BL/6J mice

Body weight of the three different treatment groups was monitored over 30 weeks. Initial weight at 4 weeks of age (start of the feeding period) was similar in all groups (initial weights of CD group: 16.1±0.7 g, HFD group: 14.5±0.6 g, HFD/CD group: 15.8±0.8 g). After 15 weeks of feeding period body weight was similarly increased in the three treatment groups (CD group: 28.9±0.5 g, HFD group: 31.2±0.7 g, HFD/CD group: 28.7±0.7 g). At the end of 30 weeks of feeding period body weight increased in

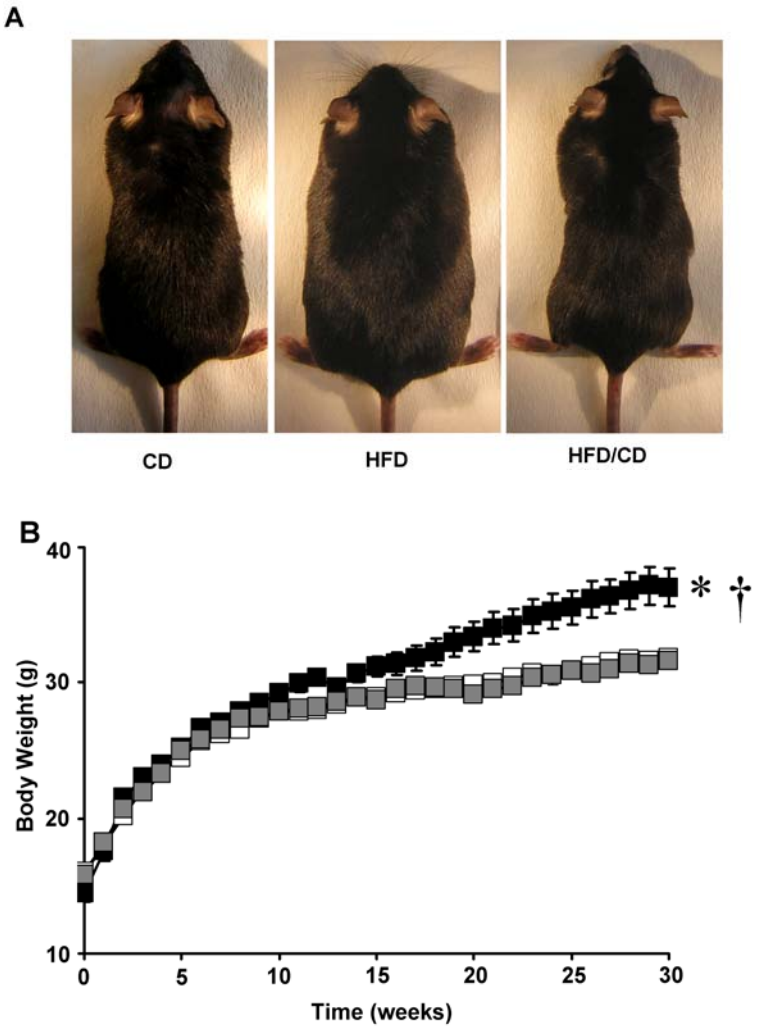

Figure 3-1 Appearance and weight gain of mice from the CD, HFD and HFD/CD group
(A) Representative photographs of mice from the CD, HFD and HFD/CD group. **(B)** Body weight was monitored weekly over 30 weeks in mice of the CD (open squares), HFD (black squares) and HFD/CD (grey squares) group. n=21-26 animals/group. Data represent means±SEM. * $p<0.05$ vs. weight of CD group (20-30 weeks), †$p<0.05$ vs. weight of HFD/CD group (20-30 weeks).

all the groups, but mice in the HFD group gained significantly more weight compared to the CD and HFD/CD group (final weight of HFD group: 37.0±1.4 g vs. CD group: 31.6±0.6 g and HFD/CD group: 31.4±0.7 g). Interestingly, final body weight of mice in the HFD/CD group was at a similar level compared to the CD group (**Figure3-1A,B**). Basal glucose levels (0 min) were similar across all groups after overnight starvation (CD group: 5.5±0.4 mM; HFD group: 6.6±0.2 mM; HFD/CD group: 6.4±0.3 mM). After 15 minutes of D-glucose injection, blood glucose levels in all the groups were higher compared to 0 min (**Figure 3-2**). At 30 minutes, glucose levels peaked in the HFD group (24.2±1.2 mM) whereas glucose concentrations in CD (16.5±1 mM) and HFD/CD group (18.6±0.7 mM) started to decrease. Furthermore, glucose concentration in mice of the HFD group did not return to the basal glucose level even after 120 minutes (HFD group: 13.6±1.3 mM vs CD group: 7.6±0.3 mM, and HFD/CD group: 8±0.4 mM) whereas glucose concentrations were similar in CD and HFD/CD group after 120 minutes (CD group: 7.6±0.3 mM, HFD/CD group: 8±0.4 mM) (**Figure 3-2**).

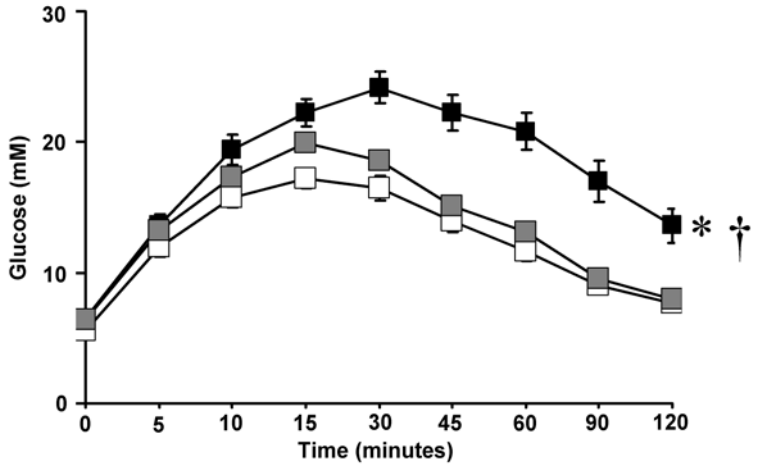

Figure 3-2 Glucose tolerance measurements of mice from the CD, HFD and HFD/CD
Concentration of blood glucose in CD (open squares), HFD (black squares) and HFD/CD (grey squares) group was measured over 120 minutes after peritoneal injection of 2 mg/g body weight of D-glucose. n=11-22 animals/group. Values represent means ± SEM.*p<0.05 vs. CD group (30-120 minutes), †p<0.05 vs. HFD/CD group (30-120 minutes).

3.2 Effect of diets on vascular reactivity

3.2.1 Vascular responses to vasoconstrictors phenylephrine and 5-hydroxytryptamine

Phenylephrine-induced contraction was investigated in thoracic aorta from mice of different dietary groups. HFD increased phenylephrine-induced contractions compared to CD (CD group: 21±3.7 %; HFD group: 46.5±5.8 %). Interestingly, contractile responses to phenylephrine remained even higher elevated by HFD/CD (HFD/CD

group: 56.7±6.6 %) (**Figure 3-3A**). **Figure 3-3B** represents contractions induced by 5-hydroxytryptamine exposure. Only HFD/CD increased 5-hydroxytryptamine-induced contractions, but not HFD (CD group: 58.8±5.4 %, HFD group: 53.1±4.6 %, HFD/CD group: 67.6±3.9 %). The effect of HFD and HFD/CD on phenylephrine-mediated contractions was more prominent than on contractions to 5-hydroxytryptamine.

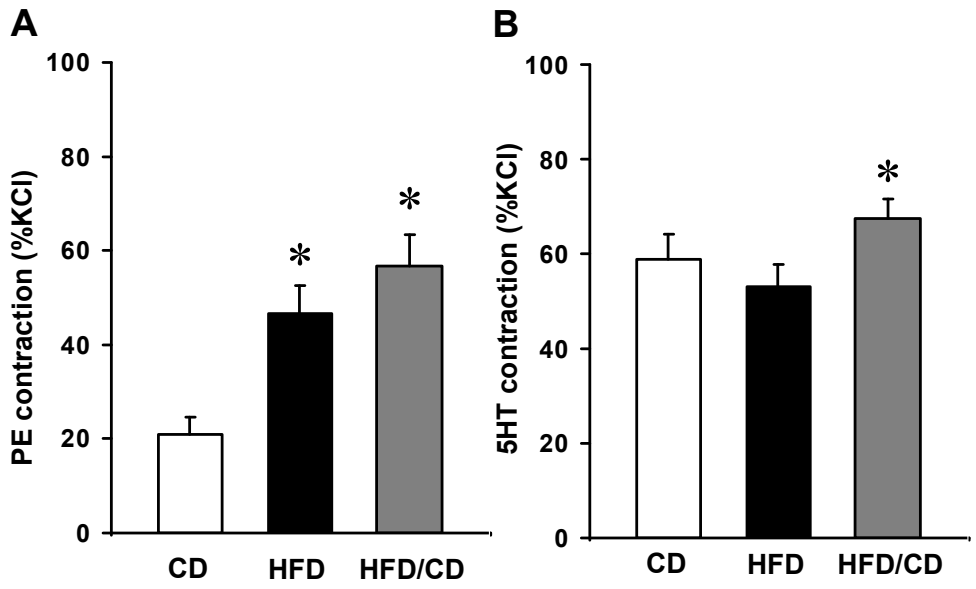

Figure 3-3 Contractile responses to phenylephrine and 5-hydroxytryptamine in thoracic aorta of mice in CD, HFD and HFD/CD group
Phenylephrine (100 nM) **(A)** and 5-hydroxytryptamine (10 µM)-induced contractions **(B)** in aortic rings are shown. Open bars represent CD group, black bars; HFD group, grey bars; HFD/CD group. Data are shown as a percentage of KCl-induced contraction. n= 4-16 animals/group. Bars represent means±SEM. *p<0.05 vs. CD.

3.2.2 Acetylcholine-induced endothelium-dependent relaxation and contraction

Endothelium-dependent relaxation was analyzed in rings of thoracic aorta from experimental groups using acetylcholine. HFD and HFD/CD had no effect on acetylcholine-induced vascular relaxation (CD group: -85.1±3.6 %, HFD group: -86.0±3.0 %, HFD/CD group: -81.3±5.1 %) (**Figure 3-4A**) and no difference in the half maximal effective concentration (EC_{50}) response to acetylcholine was observed (data not shown). Under NO-depleted condition using the NO-synthase inhibitor L-NAME, addition of acetylcholine completely blocks endothelium-dependent relaxation and induces endothelium-dependent contractions (Vanhoutte & Tang, 2008). HFD increased these contractions by 2.6 fold (HFD group: 40.3±5.1 % vs. CD group: 15.4±3.3 %) whereas by HFD/CD contractions were at a level similar compared to the CD group (HFD/CD: 11±2.8 % vs. CD group: 15.4±3.3 %) (**Figure 3-4B**).

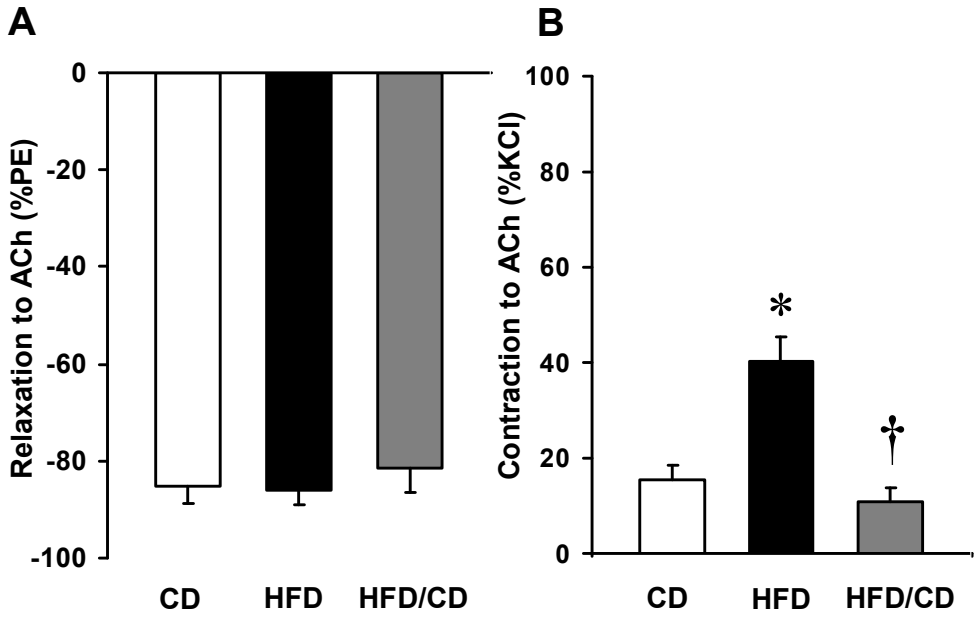

Figure 3-4 Acetylcholine-induced endothelium-dependent relaxation and contraction in thoracic aorta of mice in the CD, HFD and HFD/CD group
Thoracic aortic rings were treated in the absence **(A)** or presence **(B)** of L-NAME (300 µM) before rings were pre-contracted with cumulative concentrations of phenylephrine (0.1-100 nM) followed by acetylcholine treatment (0.1 nM-30 µM). Acetylcholine-induced relaxation at 30 µM **(A)** and contraction at 1 µM **(B)** are represented. Open bars represent CD group, black bars; HFD group, grey bars; HFD/CD group. Data are shown as a percentage of phenylephrine-induced pre-contraction **(A)** or KCl-induced contraction **(B)**. n= 8-15 animals/group. Bars represent means±SEM. * $p<0.05$ vs. CD, † $p<0.05$ vs. HFD.

3.3 Vascular gene expression analysis in dietary treatment groups

3.3.1 Gene expression analysis

Based on these physiological, metabolic and vascular functional changes induced by dietary fat intake in mice we speculated that these functional effects may be a result of changes in vascular gene expression levels and/ or formation of alternatively spliced transcripts in the vasculature. Data obtained from the DNA Mouse Exon 1.0 ST Array were compared using one-way ANOVA to identify genes that show gene expression change in any of the experimental groups. The analysis revealed only very few genes with small expressional changes within the analyzed groups. The first list ("Top Genes") includes 37 genes with gene expressional changes between any of the groups ($p< 0.01$, $\log_2$ effect (fold change) > 0.5, **Table 3-1**. Highest fold change differences between any of the groups was identified for *Cyp2E1*.

Ensembl accession number	Gene Symbol	Gene Description	log2 Effect (Fold change)	pValue
ENSMUSG00000025479	Cyp2E1	cytochrome P450, family 2, subfamily E, polypeptide 1	2.194	0.001485
ENSMUSG00000046957	Spz1	spermatogenic Zip 1	1.02	0.00168
ENSMUSG00000065610	mmu-mir-29a	mmu-mir-29a	0.8982	0.004114
ENSMUSG00000075096	Olfr1228	olfactory receptor 1228	0.8727	0.009427
ENSMUSG00000051503	EG625424	predicted gene, EG625424	0.8182	0.00615
ENSMUSG00000026691	Fmo3	flavin containing monooxygenase 3	0.8135	0.009777
ENSMUSG00000056706	Krtap7-1	keratin associated protein 7-1	0.7405	0.006657
ENSMUSG00000068646	Olfr1279	olfactory receptor 1279	0.7398	0.0007183
ENSMUSG00000030194	Tas2r116	taste receptor, type 2, member 116	0.7211	0.005026
ENSMUSG00000054072	Iigp1	interferon inducible GTPase 1	0.703	0.004058
ENSMUSG00000076559	IGKV4-54	Immunoglobulin Kappa light chain V gene segment	0.7024	0.00979
ENSMUSG00000004151	Etv1	ets variant gene 1	0.6987	0.008023
ENSMUSG00000061653	V1rb8	vomeronasal 1 receptor, B8	0.6987	0.007414
ENSMUSG00000052908	Olfr699	olfactory receptor 699	0.6836	0.001096
ENSMUSG00000070411	NM_001011810.1	olfactory receptor 485 (Olfr485), mRNA	0.6742	0.001378
ENSMUSG00000072892	Zkscan17	zinc finger with KRAB and SCAN domains 17	0.6631	0.0007077
ENSMUSG00000038576	Susd4	sushi domain containing 4	0.6629	0.007462
ENSMUSG00000040134	Rdh7	retinol dehydrogenase 7	0.6562	0.005386
ENSMUSG00000029255	Gnrhr	gonadotropin releasing hormone receptor	0.6389	0.002552
ENSMUSG00000050772	Olfr1125	olfactory receptor 1125	0.6299	0.005434
ENSMUSG00000029088	Kcnip4	Kv channel interacting protein 4	0.6275	0.00946
ENSMUSG00000031465	Angpt2	angiopoietin 2	0.6227	0.002501
ENSMUSG00000069867	Pabpnl1	poly(A)binding protein nuclear-like 1	0.5928	0.004266
ENSMUSG00000048504	Olfr453	olfactory receptor 453	0.5918	0.0008339
ENSMUSG00000047720	4922502D21Rik	RIKEN cDNA 4922502D21 gene	0.5803	0.005862
ENSMUSG00000058079	Olfr605	olfactory receptor 605	0.5799	0.008747
ENSMUSG00000030544	Mesp1	mesoderm posterior 1	0.5795	0.005686
ENSMUSG00000008686	Zfp422-rs1	zinc finger protein 422, related sequence 1	0.5785	0.006022
ENSMUSG00000002661	Alkbh7	alkB, alkylation repair homolog 7 (E. coli)	0.5529	0.006323
ENSMUSG00000010592	Dazl	deleted in azoospermia-like	0.5493	0.009727
ENSMUSG00000047976	Kcna1	potassium voltage-gated channel, shaker-related subfamily, member 1	0.538	0.007859
ENSMUSG00000061186	Sfmbt2	Scm-like with four mbt domains 2	0.5327	0.007728
ENSMUSG00000038305	2810022L02Rik	RIKEN cDNA 2810022L02 gene	0.512	0.006317
ENSMUSG00000020108	Ddit4	DNA-damage-inducible transcript 4	0.511	0.002309
ENSMUSG00000049507	4930429B21Rik	RIKEN cDNA 4930429B21 gene	0.5061	0.008182
ENSMUSG00000053088	NP_001098114.1	vomeronasal receptor Vmn2r53	0.5046	0.0009767
ENSMUSG00000020642	Rnf144a	ring finger protein 144A	0.5017	0.007075

Table 3-1 "Top Genes" list including 37 genes which show gene expression differences in any of the groups.
Content of the table shows Ensembl accession number, gene symbols of identified genes, gene description, $\log_2$ effect (fold change) and p value. Genes are ranked hierarchically based on highest $\log_2$ effect. p value and $\log_2$ effect threshold were set at < 0.01 and >0.5, respectively.

3.3.2 Exon expression analysis

Exon expression analysis was used to identify how many exons of a certain gene are significantly differentially expressed. The second list ("Top Genes by Exon") generated with one-way ANOVA includes 46 genes in which at least 2 exons of one gene show a significant expressional change in any of the three groups analyzed ($p < 0.65$, $\log_2$ effect (fold change) >0.3, **Table 3-2**. Again Cyp2E1 was identified as the top differentially expressed gene.

Ensembl accession number	Gene Symbol	Gene Description	Number of total exons	Significant probe sets	log2 Effect (Fold Change)	pValue
ENSMUSG00000025479	Cyp2E1	cytochrome P450, family 2, subfamily E, polypeptide 1	9	10	2.194	0.001485
ENSMUSG00000036815	Dpp10	dipeptidylpeptidase 10	26	19	1.681	0.01487
ENSMUSG00000027559	Car3	carbonic anhydrase 3	7	2	1.451	0.2124
ENSMUSG00000029273	Sult1d1	sulfotransferase family 1D, member 1	9	2	1.249	0.04416
ENSMUSG00000030278	Cidec	cell death-inducing DFFA-like effector c	6	3	0.985	0.0579
ENSMUSG00000030787	Lyve1	lymphatic vessel endothelial hyaluronan receptor 1	6	4	0.9668	0.01168
ENSMUSG00000019577	Pdk4	pyruvate dehydrogenase kinase, isoenzyme 4	11	5	0.9375	0.03672
ENSMUSG00000057722	Lepr	leptin receptor	22	4	0.9245	0.1302
ENSMUSG00000027445	Cst9	cystatin 9	3	2	0.9238	0.09917
ENSMUSG00000020787	P2rx1	purinergic receptor P2X, ligand-gated ion channel, 1	12	2	0.9156	0.0597
ENSMUSG00000028883	Sema3a	semaphorin 3A	24	4	0.9044	0.178
ENSMUSG00000019874	Fabp7	fatty acid binding protein 7, brain	4	2	0.8935	0.1164
ENSMUSG00000066687	Zbtb16	zinc finger and BTB domain containing 16	7	3	0.8728	0.1247
ENSMUSG00000003974	Grm3	glutamate receptor, metabotropic 3	8	2	0.8657	0.2918
ENSMUSG00000030790	Adm	adrenomedullin	4	2	0.8562	0.023
ENSMUSG00000048337	Ppyr1	pancreatic polypeptide receptor 1	2	2	0.833	0.02239
ENSMUSG00000027513	Pck1	phosphoenolpyruvate carboxykinase 1, cytosolic	8	3	0.8217	0.04389
ENSMUSG00000024222	Fkbp5	FK506 binding protein 5	11	2	0.8148	0.1045
ENSMUSG00000026691	Fmo3	flavin containing monooxygenase 3	9	2	0.8135	0.009777
ENSMUSG00000052374	Actn2	actinin alpha 2	21	2	0.7898	0.5399
ENSMUSG00000031099	Smarca1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily 1	26	2	0.7438	0.3686
ENSMUSG00000030935	Acsm3	acyl-CoA synthetase medium-chain family member 3	16	2	0.7361	0.1175
ENSMUSG00000020493	Prr11	proline rich 11	10	2	0.7172	0.1371
ENSMUSG00000020524	Gria1	glutamate receptor, ionotropic, AMPA1 (alpha 1)	18	4	0.7043	0.06306
ENSMUSG00000062991	Nrg1	neuregulin 1	9	2	0.6886	0.2022
ENSMUSG00000061723	Tnnt3	troponin T3, skeletal, fast	19	2	0.6689	0.06842
ENSMUSG00000056004	9630031F12Rik	RIKEN cDNA 9630031F12 gene	22	2	0.6327	0.415
ENSMUSG00000028033	Kcnq5	potassium voltage-gated channel, subfamily Q, member 5	19	3	0.6209	0.01316
ENSMUSG00000011463	NP_083982.1	carboxypeptidase B1	12	2	0.6103	0.4113
ENSMUSG00000022861	Dgkg	diacylglycerol kinase, gamma	25	4	0.5958	0.3253
ENSMUSG00000032254	Kif23	kinesin family member 23	24	2	0.5844	0.05934
ENSMUSG00000015879	9330182L06Rik	RIKEN cDNA 9330182L06 gene	16	2	0.5512	0.1411
ENSMUSG00000033282	Rpgrip1l	Rpgrip1-like	26	3	0.5448	0.1275
ENSMUSG00000038665	NP_001074675.	diacylglycerol kinase, iota	39	2	0.5231	0.5855
ENSMUSG00000039783	Kmo	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	17	2	0.5184	0.2477
ENSMUSG00000049482	2310061F22Rik	RIKEN cDNA 2310061F22 gene	8	3	0.515	0.4793
ENSMUSG00000045328	Cenpe	centromere protein E	46	2	0.4955	0.3941
ENSMUSG00000040084	Bub1b	budding uninhibited by benzimidazoles 1 homolog,beta (S. cerevisiae)	23	2	0.4751	0.1324
ENSMUSG00000019359	Gdpd2	glycerophosphodiester phosphodiesterase domain containing 2	16	2	0.4725	0.269
ENSMUSG00000027985	Lef1	lymphoid enhancer binding factor 1	13	2	0.4668	0.6247
ENSMUSG00000026141	Col19a1	collagen, type XIX, alpha 1	53	2	0.451	0.3487
ENSMUSG00000014850	Msh3	mutS homolog 3 (E. coli)	26	2	0.4494	0.1209
ENSMUSG00000033826	Dnahc8	dynein, axonemal, heavy chain 8	93	3	0.4492	0.08218
ENSMUSG00000022262	Dnahc5	dynein, axonemal, heavy chain 5	79	2	0.4163	0.333
ENSMUSG00000057378	Ryr3	ryanodine receptor 3	104	2	0.3997	0.2306
ENSMUSG00000022483	Col2a1	collagen, type II, alpha 1	56	2	0.3742	0.2631

Table 3-2 "Top Genes by Exon" list including 46 genes in which at least 2 exons show a significant expression difference in any of the three groups
Content of the table shows Ensembl accession number, gene symbols of identified genes, gene description, number of total exons of the gene, significant probe sets (exons) of the identified genes, $\log_2$ effect (fold change) and p value. Genes are ranked hierarchically based on highest $\log_2$ effect. p value and $\log_2$ effect threshold was set at < 0.65 and >0.3, respectively.

3.3.3 Gene Ontology classification

The 46 identified genes from the "Top Genes by Exon" list were classified by Gene Ontology (GO) using GeneGO Metacore software (GeneGO, Inc.) to assign biological meaning. The top most 10 significant GO processes are shown in **Figure 3-5**. Analysis determined GO processes of genes with a minimum $\log_2$ effect (fold change) of 0.3 and a maximum p value of 0.06. Results revealed that genes cluster mainly to biological processes involved in regulation of vasoconstriction, circulatory system process, blood circulation, and positive regulation of vasodilatation.

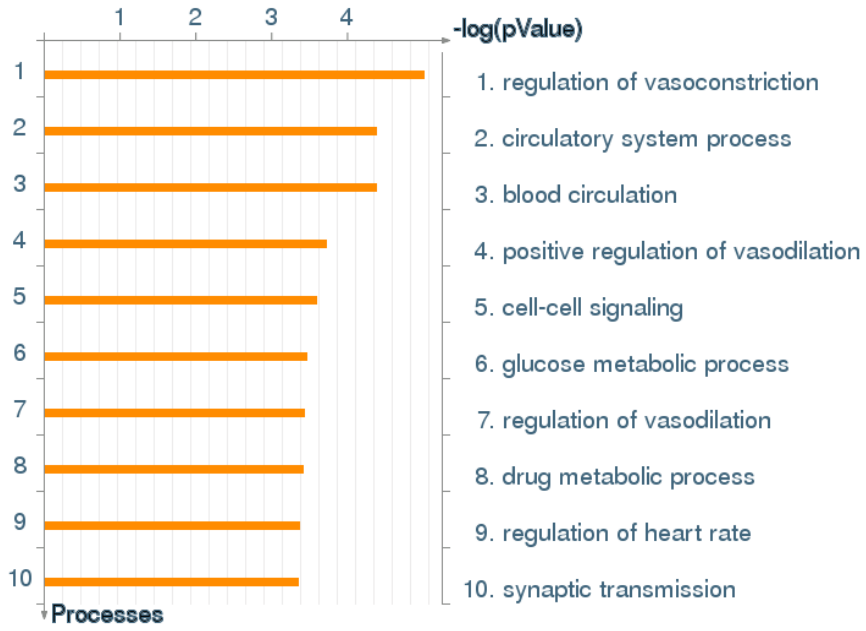

Figure 3-5 Gene Ontology Classification by GeneGO Metacore
Identified genes from the "Top Genes by Exon" list are grouped in regard to biological processes. Top 10 biological processes are listed sorted by the lowest p value. Longer orange bars indicate lower p value for genes in a GO category. Figure was generated using GeneGO Metacore software (GeneGO Inc).

3.3.4 Visualization of probe set expression for selected target genes

Probe set expression as measured by intensity values of genes were visualized as line graphs. **Figure 3-6** and **Figure 3-7** show representative line graphs of 12 genes chosen for further validation based on the criterion to be changed by HFD and HFD/CD. The line graphs illustrate differences in intensity values of every probe set between the treatment groups. Depending on the length, an exon is covered by several probe sets. Short exons are not covered by a probeset (Appendix **Table 5-3**)

This is the case for exons 17 and 24 of diacylglycerol kinase, gamma (*Dgkg*), exons 15 and 17 of dipeptidylpeptidase (*Dpp10*), exon 1 of flavin containing monooxygenase (*Fmo3*), exons 14, 17, and 21 for leptin receptor (*Lepr*).

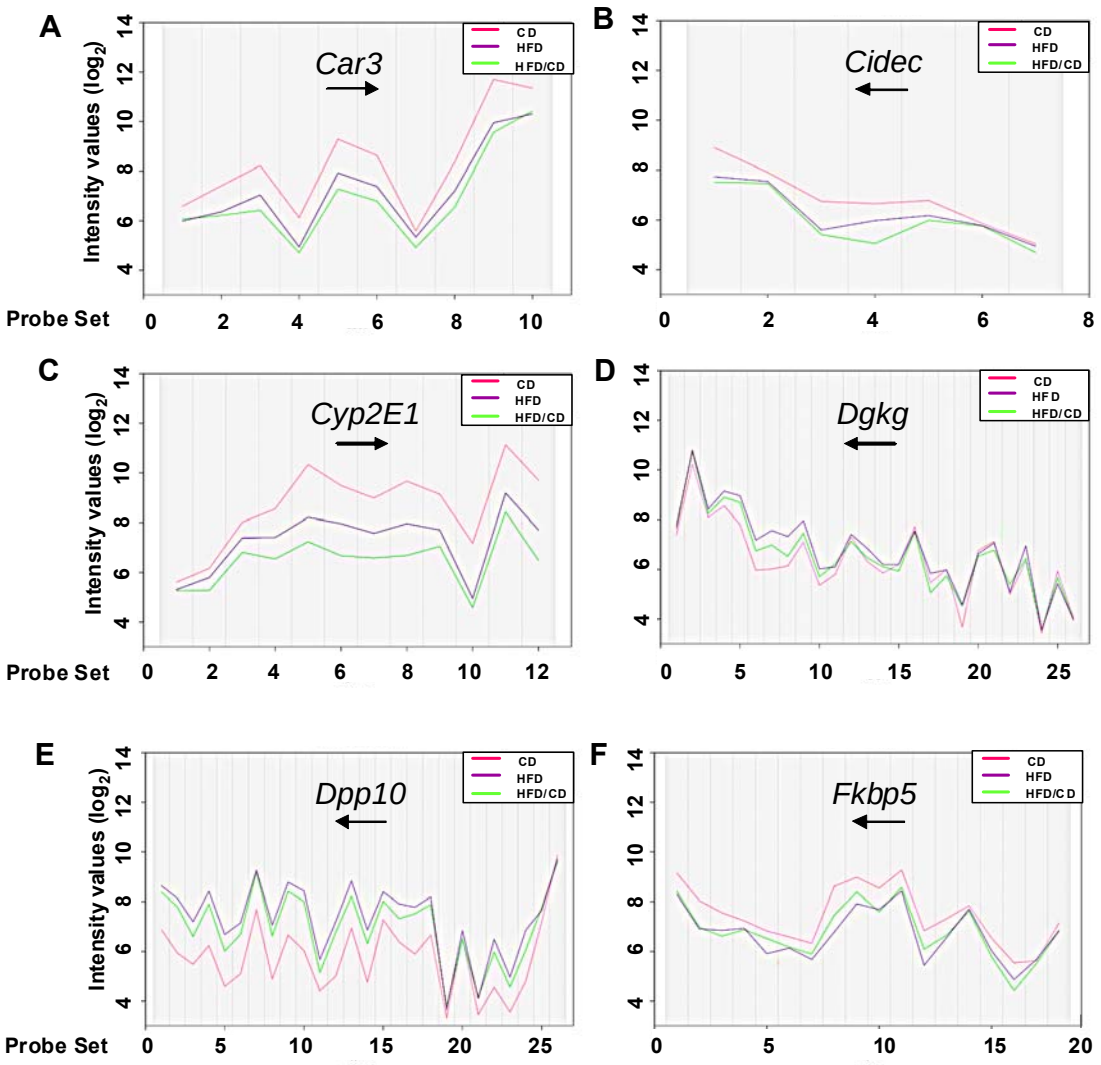

Figure 3-6 Visualization of probe set/exon intensity values of target genes
Each Intensity value (log_2) on the y-axis is plotted against the total number of probe sets of the respective gene (*Car3*, carbonic anhydrase 3*; Cidec*, cell-death-inducing DFFA-like effector c; *Cyp2E1*, cytochrome P450, family 2, subfamily E, polypeptide 1; *Dgkg*, diacylglycerol kinase gamma; *Dpp10*, dipeptidylpeptidase 10; and *Fkbp5*, FK506 binding protein 5) on the x-axis. Arrow indicates 5`to 3`direction of the gene. CD group (red line), HFD group (purple line), and HFD/CD group (green line).

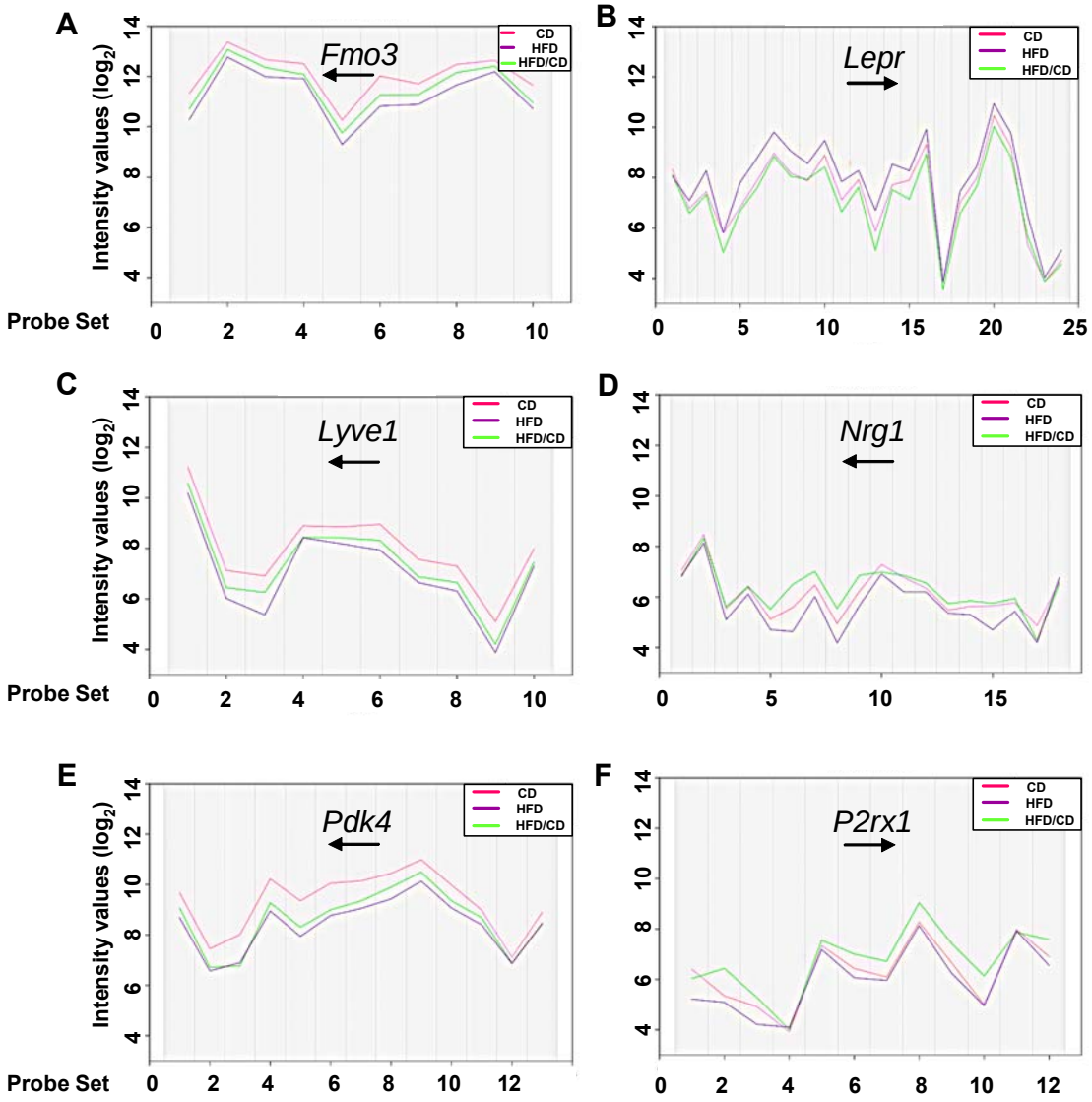

Figure 3-7 Visualization of probe set/exon intensity values of target genes
Each Intensity value (log_2) on the y-axis is plotted against the total number of probe sets of the respective gene (*Fmo3*, flavin containing monooxygenase 3; *Lepr*, leptin receptor; *Lyve1*, lymphatic vessel endothelial hyaluron receptor 1; *Nrg1*, neuregulin 1; *Pdk4*, pyruvate dehydrogenase kinase, isoenzyme 4; and *P2rx1*, purigenic receptor P2X ligand-gated ion channel) on the x-axis. Arrow indicates 5`to 3`direction of the gene. CD group (red line), HFD group (purple line), and HFD/CD group (green line).

3.4 Validation of selected target genes

To further investigate whether the expression of the 12 chosen genes in aortic tissue are specifically and sustainably changed by HFD and HFD/CD, steady state mRNA expression levels were validated with gene specific mouse primer pairs (Appendix **Table 5-1**) using qRT-PCR. **Table 5-1** also shows which exons of selected genes are covered by primer pairs.

3.4.1 Genes with no expressional change

Dietary treatments had no effect on steady-state mRNA expression levels of the genes FK506 binding protein 5 (*Fkbp5*) (**Figure 3-8A**), *Fmo3* (**Figure 3-8B**), neuregulin 1 (*Nrg1*) (**Figure 3-8C**), lymphatic vessel endothelial hyaluronan receptor 1 (*Lyve1*) (**Figure 3-8D**), pyruvate dehydrogenase kinase, isoenzyme 4 (*Pdk4*) (**Figure 3-8E**), and purinergic receptor P2X, ligand-gated ion channel 1 (P2rx1) (**Figure 3-8F**).

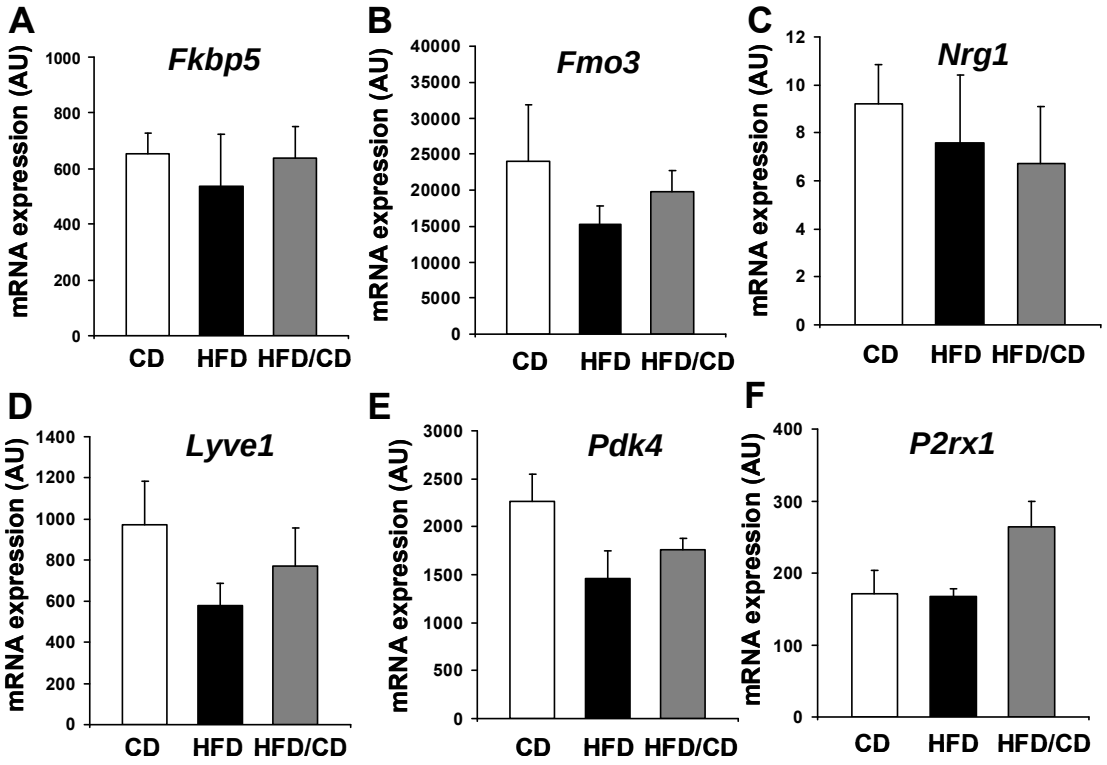

Figure 3-8 Genes that are not changed by different diets
mRNA expression levels of target genes *Fkbp5* **(A)**, *Fmo3* **(B)**, *Nrg1* **(C)**, *Lyve1* **(D)**, *Pdk4* **(E)** and *P2rx1* **(F)** induced by the different dietary treatments are represented. mRNA expression was calculated by ΔCT of the gene of interest and the housekeeping gene β-actin =arbitrary units (AU). Values represent means ± SEM. Open bars, CD; black bars, HFD; grey bars, HFD/CD. n=5-6 aortic samples/experimental group.

3.4.2 Changes in gene expression levels induced by high-fat diet

Steady state mRNA expression levels of *Lepr* (**Figure 3-9A**) and *Dgkg* (**Figure 3-9B**) were upregulated approximately 2-fold by HFD and expression levels in HFD/CD group were similar to CD group.

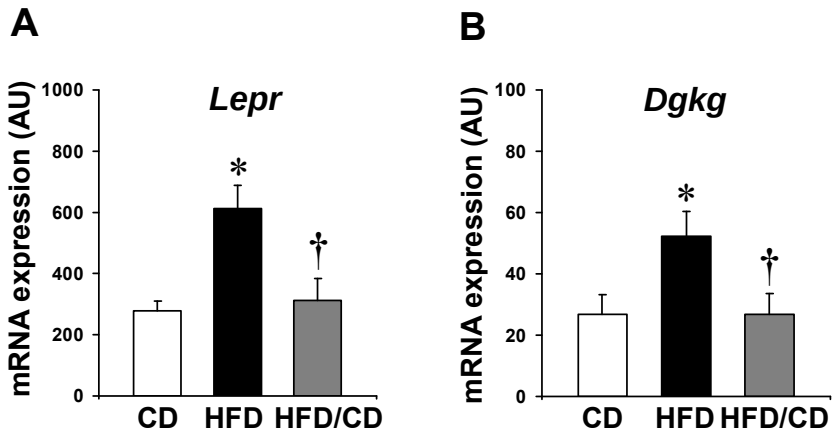

Figure 3-9 Validation of genes that are changed by HFD but not by HFD/CD
mRNA expression levels of target genes *Lepr* **(A)** and *Dgkg* **(B)** induced by the different dietary treatments are represented. mRNA expression was calculated by ΔCT of the gene of interest and the housekeeping gene β-actin and expressed in arbitrary units (AU). Values represent means ± SEM. Open bars, CD; black bars, HFD; grey bars, HFD/CD. n=5-6 aortic samples/experimental group.* $p<0.05$ vs. CD, † $p<0.05$ vs. HFD.

3.4.3 Expression levels of genes with "high-fat diet memory effect"

Analysis of data of the qRT-PCR showed that steady state mRNA expression of *Dpp10*, carbonic anhydrase 3 (*Car3*), *Cidec*, and cytochrome P450, family2, subfamily E, polypeptide 1 (*Cyp2E1*) were specifically and sustainably changed by HFD and HFD/CD (**Figure 3-10**). *Dpp10* gene expression was 2.4 fold upregulated in the HFD group and 3.4 fold in the HFD/CD group compared to the CD group (**Figure 3-10A**) Expression of *Car3* and *Cidec* was downregulated by HFD (*Car3* and *Cidec*: 3.8 fold) and HFD/CD (*Car3*: 3.1 fold and *Cidec*: 2.6 fold) (**Figure 3-10B,C**). Expression of *Cyp2E1* was downregulated by 3.6 fold in the HFD group and 4.8 fold in the HFD/CD group (**Figure 3-10D**).

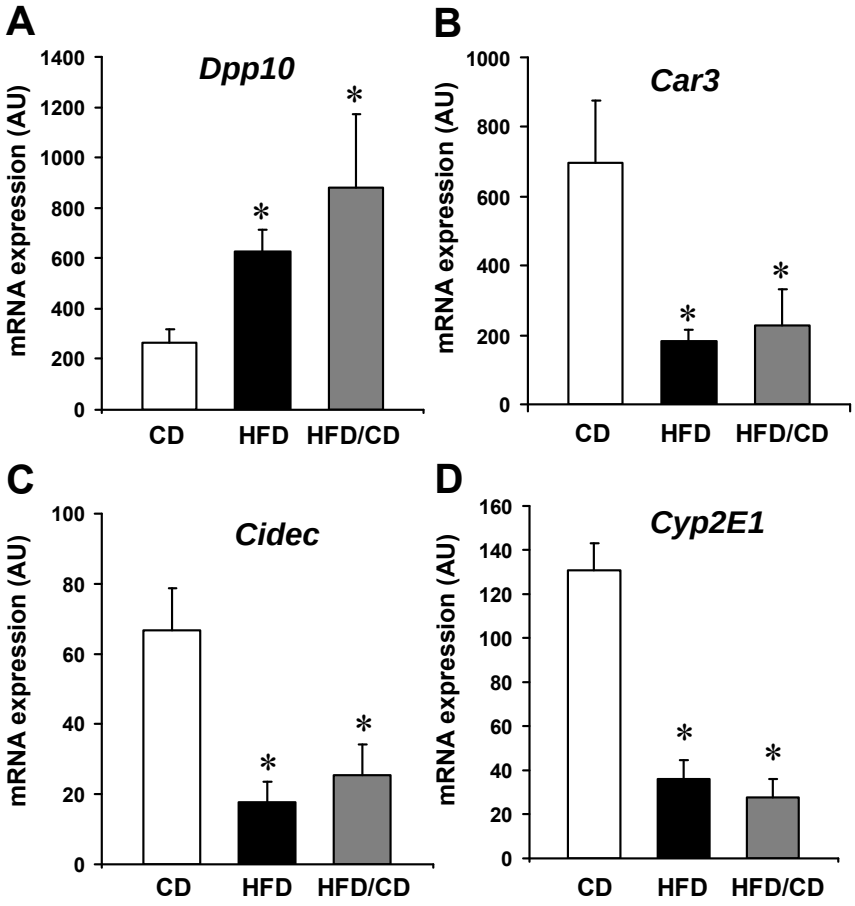

Figure 3-10 Validation of genes that are changed by HFD and HFD/CD
mRNA expression levels of target genes *Dpp10* **(A)**, *Car3* **(B)**, *Cidec* **(C)** and *Cyp2E1* **(D)** induced by the different dietary treatments are represented. mRNA expression was calculated by ΔCT of the gene of interest and the housekeeping gene β-actin =arbitrary units (AU). Values represent means ± SEM. Open bars, CD; black bars, HFD; grey bars, HFD/CD. n=5-6 aortic samples/experimental group for qRT-PCR. * $p<0.05$ vs. CD.

3.4.4 Protein expression level analysis of genes with "high-fat diet memory effect"

To determine protein expression levels of aortic Car3, Cidec and Cyp2E1 in different dietary treatment groups, immunoblotting was performed using specific antibodies for Car3, Cidec and Cyp2E1. Extracellular signal-regulated kinase 1 and 2 (Erk1/2) antibody was used as loading control. HFD and HFD/CD downregulated Car3 protein expression level which is in line with mRNA expression levels (**Figure 3-11A**). Protein expression level of Cidec was upregulated by HFD and HFD/CD whereas mRNA expression was downregulated (**Figure 3-11B**).

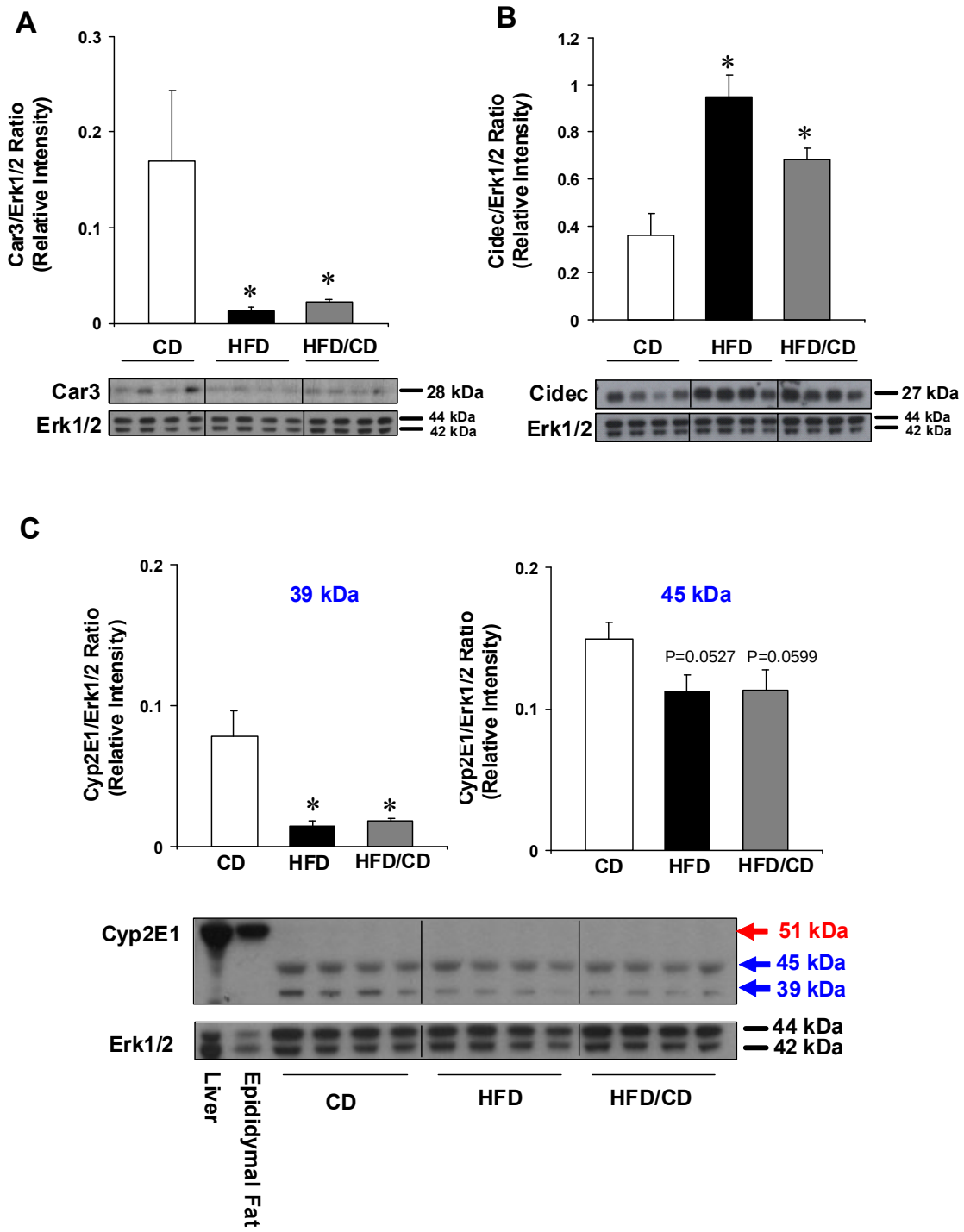

Figure 3-11 Protein expression levels of Car3, Cidec and Cyp2E1 in aortic tissue of mice undergoing different dietary protocols

Car3 **(A)** Cidec **(B)** and Cyp2E1 **(C)** in CD, HFD and HFD/CD group were analyzed using Western blot. Car3 is detectable at 28 kDa **(A)** and Cidec **(B)** at 27 kDa using the respective antibodies. Erk1/2 antibody was used as a loading control. Total protein lysates from liver and epididymal fat were used as positive controls to detect full-length Cyp2E1 at 51 kDa (red arrow). Blue arrows at 39 and 45 kDa indicate the size of Cyp2E1 protein in aorta **(C; lower panel)**. Densitometry quantification of aortic Cyp2E1 protein at 39 **(C; upper, left panel)** and 45 kDa **(C; upper, right panel)** is shown. Values represent means ± SEM. Open bars, CD; black bars, HFD; grey bars, HFD/CD. n=4 aortic samples for immunoblotting.* $p<0.05$ vs. CD.

Immunoblotting analysis using the anti-Cyp2E1 (rabbit polyclonal antibody against full-length Cyp2E1) showed that full-length Cyp2E1 is highly expressed in liver and epididymal fat (both tissues were used as positive controls), and is detectable at 51 kDa while in aorta Cyp2E1 is expressed at low level and detected at 39 and 45 kDa in all the groups, but not at 51 kDa (**Figure 3-11C, lower panel**).This expression level pattern was confirmed also with a different antibody (rabbit polyclonal antibody against a synthetic peptide corresponding to C-terminal Cyp2E1, data not shown). Densiometric analysis of the blots revealed that aortic Cyp2E1 variant detected at 39 kDa was significantly downregulated 8-fold by HFD and 4-fold by HFD/CD (**Figure 3-11C**, **upper, left panel**) while Cyp2E1 variant detected at 45 kDa tended to be downregulated by 1.4-fold by HFD (p=0.0527) and HFD/CD (p=0.0599), respectively (**Figure 3-11C, upper, right panel**).

3.5 Motivation to select *Cyp2E1* for further in depth analysis

The statistical analysis of the DNA Exon array and validation with qRT-PCR was revealing *Cyp2E1* to be the top most differentially expressed gene within the groups (2.2 fold change compared to other genes which ranged between fold changes of 0.4-1.7). Cyp2E1 belongs to the Cyp450 enzymes, a family of heme-containing monooxygenases which play an important role in the metabolism of arachidonic acid AA to HETEs (Laethem *et al.*, 1993). These molecules contribute to the regulation of vascular homeostasis (Elbekai & El-Kadi, 2006). Cyp2E1 acts as ω- hydroxylase catalyzing the formation of 18 (*R*)-, 19 (*R*)-and 19(*S*)-HETEs from AA in the vasculature (Laethem *et al.*, 1993). These metabolites were shown to act as vasodilators (Zhang *et al.*, 2005; Schafer *et al.*, 2010). Furthermore, Cyp2E1 can also generate reactive oxygen species (Lu & Cederbaum, 2008) which affect vascular reactivity (Cai & Harrison, 2000). So far only little data exist on the role of Cyp2E1 in vascular function and to my knowledge nothing is known how different diets influence Cyp2E1 expression in aortic tissue. Furthermore, Western blot analysis showed two shorter protein forms of Cyp2E1 to be expressed in aorta. It is not known whether splice variants of Cyp2E1 exist in aortic tissue.

3.6 Analysis of vascular *Cyp2E1* splice variants

3.6.1 Statistical analysis of *Cyp2E1* exon-specific differences between dietary treatment groups

The *Cyp2E1* gene consists of 9 exons and nucleotide sequence of full-length *Cyp2E1* mRNA contains 1759 bp (NCBI Accession # BC013451) The DNA Mouse Exon 1.0 ST Array from Affymetrix is designed that every exon of a gene is covered by at least one probeset (**Figure 3-12**).

To assess whether *Cyp2E1* is alternatively spliced, Tukey`s post hoc test based on one-way ANOVA results of exon microarray data was applied to identify which

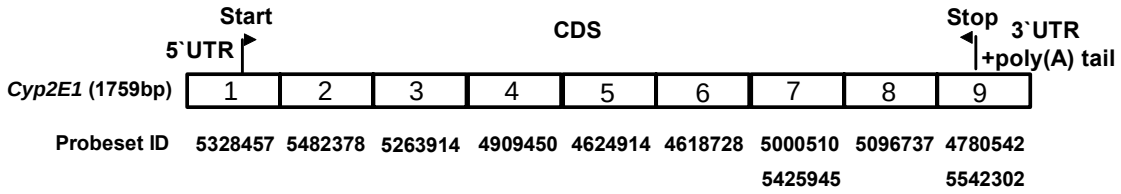

Figure 3-12 Schematic representation of full-length *Cyp2E1* transcript
Cyp2E1 consists of 9 exons (boxes) and contains a nucleotide sequence of 1759 bp. Scheme represents the location of the 5`untranslated region (5`UTR); Start, start codon of *Cyp2E1*, CDS (coding sequence) of *Cyp2E1*, Stop, stop codon of *Cyp2E1*, 3`untranslated region (3`UTR), and poly(A) tail, polyadenylation tail. DNA exon arrays have a following design: Every exon is covered by at least one probeset. To distinct which probeset covers which exon, every probe set has its own ID. Depending on the length, one exon can be covered by more than one probeset as in exon 7 and 9.

probesets (covering and representing exons) are differentially expressed between the

Group comparisons	Exon Probeset ID	young-fat effect [Tukey p-value]	Fold Change [log2 ratio]	Exon Probeset ID maches to Exon
HFD vs. CD	4909450	0.02228	-2.071	4
	5425945	0.009736	-1.443	7
	5096737	0.001632	-2.192	8
	5542302	0.01587	-1.939	9
HFD/CD vs. CD	5482378	0.01399	-1.214	2
	5263914	0.01066	-1.825	3
	4909450	0.001902	-3.043	4
	4624914	0.0108	-2.807	5
	4618728	0.006739	-2.449	6
	5000510	0.002449	-2.953	7
	5425945	0.0008094	-2.074	7
	5096737	0.0004613	-2.601	8
	4780542	0.01226	-3.211	9
	5542302	0.001907	-2.685	9

Table 3-3 Tukey`s post hoc test analysis of *Cyp2E1*
Table represents specific exons of *Cyp2E1* that are differentially expressed between HFD or HFD/CD and CD group. p value threshold was set to < 0.05 and fold change threshold to > -0.4. Negative fold change indicates downregulation.

groups. Results for *Cyp2E1* are shown in **Table 3-3** (HFD vs. CD group and HFD/CD vs. CD group). Analysis between HFD and CD group showed that 4 probe sets covering 4 different exons of *Cyp2E1* are downregulated (minimum fold change: -1.4 fold for exon 7; maximum fold change: -2.2 fold for exon 8), while comparison of HFD/CD and CD group showed that 10 probe sets covering 8 exons (2 different probe sets cover exon 7 and exon 9) of *Cyp2E1* are differentially downregulated between HFD/CD and CD group (minimum fold change: -1.2 fold for exon 2; maximum fold change: - 3.2 fold for exon 9).

3.6.1.1 Exon-specific validation of *Cyp2E1* using PCR

To investigate whether the differentially expressed exons identified for *Cyp2E1* are due to the existance of truncated exons of *Cyp2E1*, exon-spanning primers (each primer

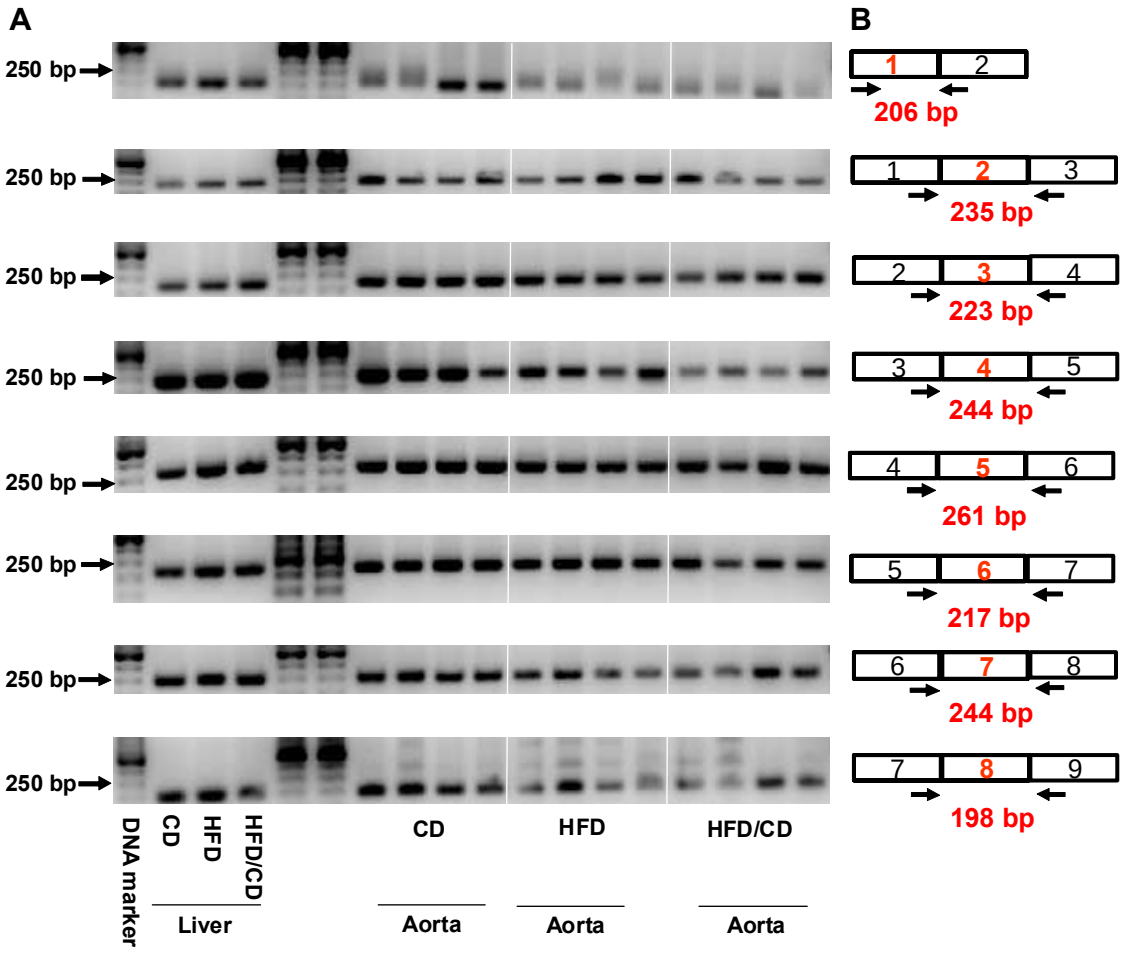

Figure 3-13 "Exon-specific" PCR of *Cyp2E1* exons 1-8 in mouse liver and aorta
(A) Illustrated are PCR amplicons generated with specific exon-spanning primer pairs for exons 1 to 8 **(B)** Scheme shows the position of the different primer pairs (forward and reverse primer, indicated by black arrows) for each exon 1-8 and corresponding PCR product sizes in red. PCR amplicons were separated on a 2 % agarose gel. A 50 bp DNA marker was used to estimate size of the PCR products. As positive control cDNA from one liver per group was used to perform PCR, while cDNA from four different mouse aortae per group was used.

pair include one exon, (Appendix **Table 5-2**) were designed (Modrek & Lee, 2002; Erkens *et al.*, 2008). Schematic representation of PCR amplicons of *Cyp2E1* exons 1-8 are shown in **Figure 3-13** "Exon-specific" PCRs revealed that exons 1-8 are expressed in aorta of all groups (**Figure 3-13A**) with variations in expression levels (verified by qRT-PCR, data not shown). The amplicon sizes from exons 1-8 (**Figure 3-13B)** matched to those of mouse liver cDNA which was used as a positive control. Total size of *Cyp2E1* exon 9 is 424 bp of which 185 bp correspond to the coding sequence including the stop codon, and 239 bp to the 3`untranslated region (3`UTR).

Analysis of exon 9 using reverse primer Exon 9/1_3`UTR (Appendix **Table 5-2**) binding at basepair position 196 in the 3`UTR of *Cyp2E1* indicated a truncation of this

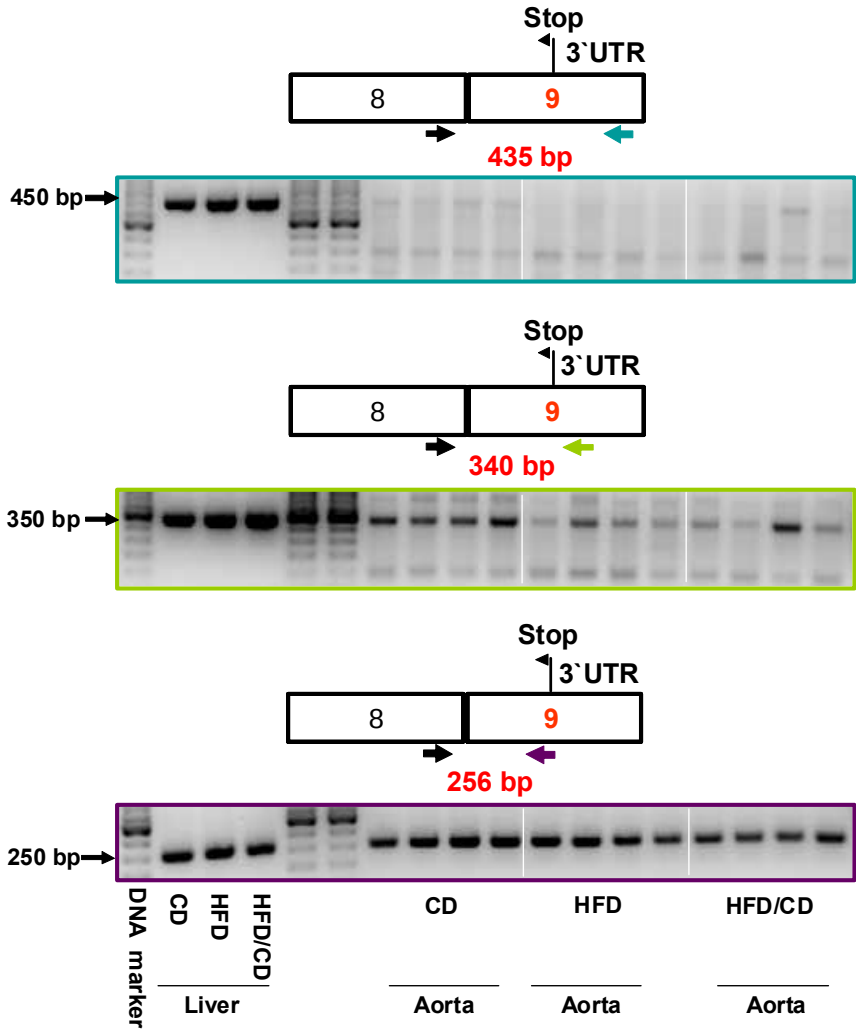

Figure 3-14 "Exon-specific" PCR of *Cyp2E1* exon 9 in mouse liver and aorta
PCR amplicons of exon 9 are represented generated with three different reverse primers (blue, green and purple arrows). Different PCR product sizes of exon 9 are indicated in red. PCR amplicons were separated on a 2 % agarose gel. A 50 bp DNA marker was used to estimate size of the PCR products. As positive control cDNA from one liver per group was used to perform PCR, while cDNA from four different mouse aortae per group was used. Stop, stop codon of *Cyp2E1*, 3`untranslated region (3`UTR).

region in aorta of all the groups, but not in liver(**Figure 3-14**, blue arrow). To narrow the region in which the truncation of exon 9 starts, two other reverse primers, Exon 9/2_3`UTR and Exon 9/3_Stop were designed (Appendix **Table 5-2**). Reverse primer Exon 9/2_3`UTR is indicated in **Figure 3-14** with a green arrow (binds at basepair position 104 in the 3`UTR of *Cyp2E1*), and reverse primer Exon 9/3_Stop is indicated by a purple arrow (**Figure 3-14**, this primer binds to the stop codon region at basepair position 185 of *Cyp2E1*) Visualization of PCR amplicons indicate that , 81 bp corresponding to the 3`UTR (distance between purple, 185 bp, and green reverse primer, 104 bp) are present in aortic tissue of all groups but amplification rate decreases with HFD and HFD/CD. Interestingly, PCR products with a size of 92 bp corresponding to the 3`UTR of exon 9 (distance between blue, 196 bp and green reverse primer, 104 bp) are not present in any of the dietary treatment groups (**Figure 3-14**).

3.6.1.2 Exon skipping analysis of aortic *Cyp2E1*

Full-length *Cyp2E1* mRNA in liver consists of 9 exons and contains a nucleotide sequence of 1759 bp with a molecular weight of ~51 kilo Dalton (kDa) for the translated protein. Two *Cyp2E1* splice variants in liver are identified (Zhuge & Cederbaum, 2006a; b). Interestingly, in both splice variants 229 bp of the 3`UTR from exon 9 are missing. In addition, variant 1 lacks exons 4-6 (NCBI Accession # DQ838976, 1012 bp, 37 kDa) while variant 2 lacks exons 1-6 and 35 bp from exon 7 (NCBI Accession # DQ838977, 484 bp, 17 kDa). To test whether known *Cyp2E1* variants are detectable in aortic tissue, PCR was performed using a forward primer binding at the end of exon 3 (ForEx3) and a reverse primer binding at the beginning of exon 8 (RevEx8, **Figure 3-15A**, Appendix) Liver cDNA was used as a positive control. Visualization of the amplicons from liver and aorta using agarose gel electrophoresis revealed an upper band corresponding to a PCR product size of approximately 850 bp and a lower band at ~ 400 bp in two of four aortic samples in the CD group (**Figure 3-15B**). In the aortic samples of the HFD and HFD/CD group, no lower band at ~ 400 bp was detectable.

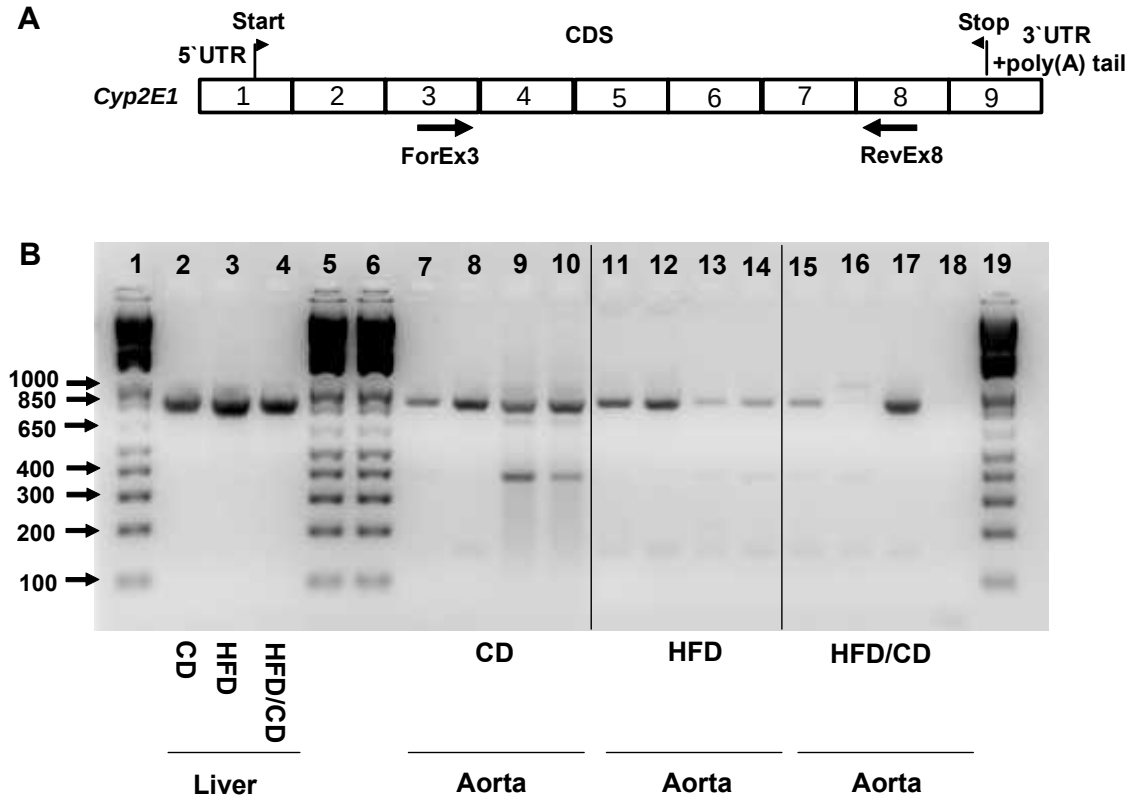

Figure 3-15 Representative agarose gel image of separated PCR products amplified using the specific primer pair ForEx3 and RevEx8 in liver and aorta of CD, HFD and HFD/CD group
(A) Scheme represents the location of the primer pair (direction of the primers indicated by black arrows) in *Cyp2E1* full-length transcript. **(B)** Amplified PCR products from all groups (CD, HFD, HFD/CD) were loaded on a 2 % agarose gel. 1 kb DNA ladder (lane 1, 5, 6, and 19) was used to estimate size of all PCR products. As positive control cDNA from one liver per group (lane 2, liver CD; lane 3, liver HFD; lane 4, liver HFD/CD) was used, while cDNA from four different mouse aortae per group (lane 7-10, aorta CD; lane 11-14, aorta HFD; lane 15-18, aorta HFD/CD) was used to perform PCR. ForEx3 (forward primer binding at exon 3); RevEx8 (reverse primer binding at exon 8); 5`UTR, 5`untranslated region; Start, start codon of Cyp2E1, CDS, coding sequence of Cyp2E1, Stop, stop codon of Cyp2E1, 3`UTR, 3`untranslated region and poly(A) tail, polyadenylation tail.

PCR bands of the CD group were excised from the agarose gel, purified and sequenced. In **Figure 3-16** aligned sequencing results are shown. Using nucleotide "Basic Local Alignment Search Tool" (BLAST® from NCBI) upper band was identified as *Cyp2E1* wild type sequence with an expected product size of 850 bp including exon 4-6 of *Cyp2E1*. Interestingly, the sequencing results for the lower band showed that exon 4-6 are skipped.

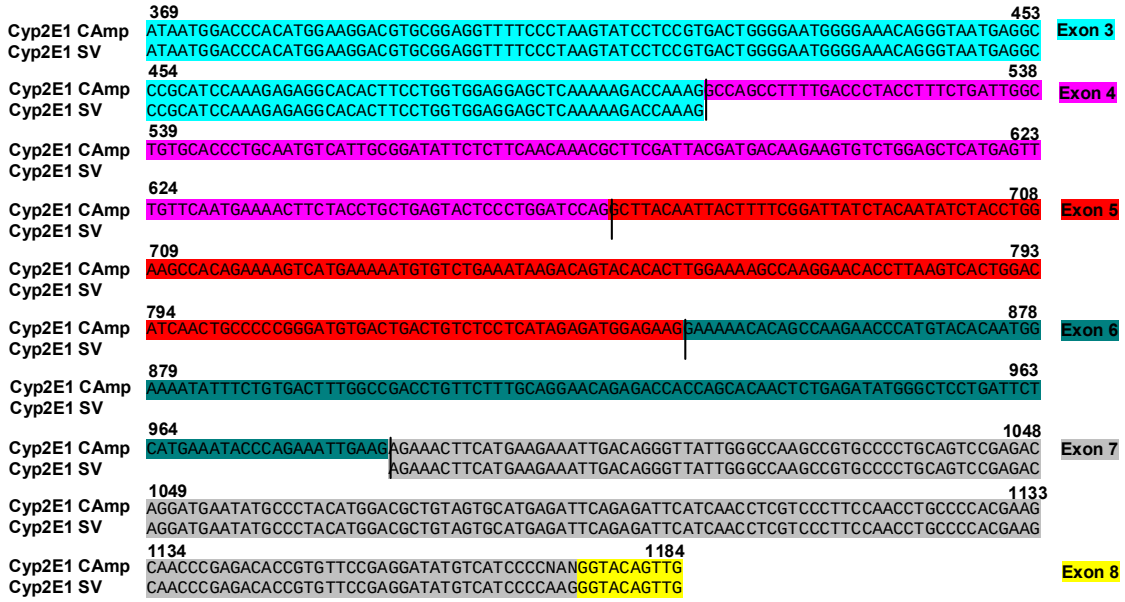

Figure 3-16 Nucleotide sequence alignment of complete *Cyp2E1* amplicon and *Cyp2E1* splice variant in aorta of the CD group
Cyp2E1 CAmp represents the nucleotide sequence of complete *Cyp2E1* amplicon whereas *Cyp2E1* SV represents the nucleotide sequence of the splice variant in which complete exon 4 (pink), exon 5 (red) and exon 6 (dark green) are spliced out. Numbers 369-1184 represent nucleotide basepair positions of complete mRNA sequence targeted by primer pair ForEx3 and RevEx8. Vertical black lines indicate beginning and end of a particular exon. Colours represent different exons.

3.7 Effect of DETC on vascular reactivity in dietary treatment groups

To investigate whether Cyp2E1 contributes to vascular reactivity in aorta of mice from the CD, HFD and HFD/CD group, the first approach was to inhibit Cyp2E1 with DETC. It is important to note that DETC inhibits Cyp2E1 in liver microsomes (Guengerich *et al.*, 1991) and arteries of hypertensive rats (Zhang *et al.*, 2005), but also SOD in aorta in mice, rats, and rabbits (Ghanam *et al.*, 1998; Karasu, 2000; Takenouchi *et al.*, 2009).

3.7.1 Impact of DETC on responses to vasoconstrictors phenylephrine and 5-hydroxytryptamine

Cumulative concentrations of phenylephrine were added after rings were incubated without or with DETC for 30 minutes. As shown previously, without addition of inhibitors, contractile responses to phenylephrine at 100 nM were significantly increased with HFD and HFD/CD (see also **Figure 3-3A**). Inhibition with DETC significantly increased phenylephrine-mediated contractions in the CD group (21±3.7 % vs. 33.3±4.1 %) (**Figure 3-17A**) and were decreased in HFD (46.5±5.8 % vs. 26.7±4.1 %) and HFD/CD group (56.7±6.6 % vs. 29.4±2.9 %) (**Figure 3-17B,C**, respectively). Contractions to phenylephrine due to inhibition with DETC were similar in all groups

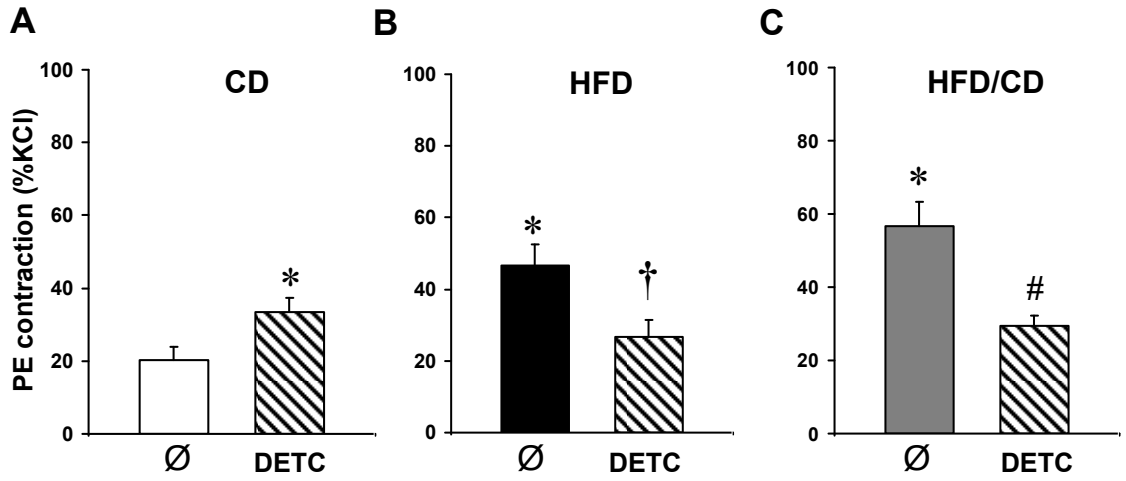

Figure 3-17 Effect of DETC on phenylephrine-mediated contraction in CD, HFD, and HFD/CD group
Phenylephrine (PE)-induced contractions were determined by incubating rings either without inhibitor (Ø) (open bar, CD; black bar, HFD; grey bar, HFD/CD) or with DETC (100 µM) (striped bars) before contracting the vessels with cumulative concentrations of phenylephrine (0.1-100 nM). Data represent phenylephrine-induced contractions at a concentration of 100 nM **(A-C)**. Data are shown as a percentage of KCl-induced contraction. n=9-20 animals/group. Values represent means±SEM.* $p<0.05$ vs. CD group Ø, † $p<0.05$ vs. HFD group Ø, # $p<0.05$ vs. HFD/CD group Ø.

(CD DETC: 33.3±4.1 %; HFD DETC: 26.7±4.1 %; HFD/CD DETC: 29.4±2.9 %).

5-hydroxytryptamine-mediated contractions were analyzed using no inhibitor or DETC in the thoracic aorta of the CD, HFD and HFD/CD group (Figure 3-18). At a concentration of 10 µM, contractions were induced in all the three groups. In the absence of DETC, only 5-hydroxytryptamine contraction in the HFD/CD group was significantly increased, but not in the HFD group (see also **Figure 3-3B**). Interestingly, addition of DETC significantly reduced 5-hydroxytryptamine-induced contractions in the CD (58.8±5.4 % vs. 33.5±4.1 %) and HFD/CD group (67.6±3.9 % vs. 46.5±5.2 %) (**Figure 3-18A,C**), but not in the HFD group (53.1± 4.6 % vs. 43.2± 7.9 %) (**Figure 3-18B**). However, contractions to 5-hydroxytryptamine in the presence of DETC were

similar in all groups (CD DETC: 33.5±4.1 %; HFD DETC: 46.5±5.2 %; HFD/CD DETC: 43.2.±7.9 %).

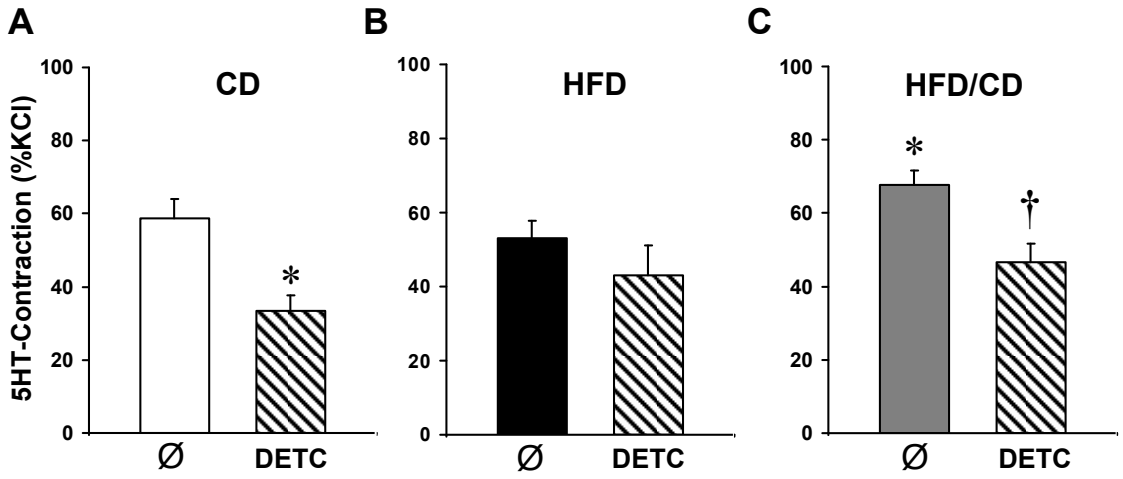

Figure 3-18 Role of DETC in 5-hydroxytryptamine-mediated contraction in CD, HFD, and HFD/CD group
Aortic rings were incubated in the absence (open bar, CD; black bar, HFD; grey bar, HFD/CD) or presence (striped bars) of DETC (100 µM) for 30 minutes before cumulative concentrations of 5-hydroxytryptamine (0.1 nM-10 µM) were added. 5-hydroxytryptamine (5-HT) contraction at a concentration of 10 µM is represented **(A-C)**. n=9-14 animals/group. Data are shown as a percentage of KCl-induced contraction. Data represent means±SEM.* $p<0.05$ vs. CD group Ø, † $p<0.05$ vs. HFD/CD group Ø.

3.7.2 Effect of DETC on endothelium-dependent contractions induced by acetylcholine

Endothelium-dependent contractions were induced under NO-depleted condition (as described in 3.2.2) at high concentration of acetylcholine (1 µM) in all the three groups (CD group: 15.4±3.3 %, HFD group: 40.3±5.1 %, HFD/CD group: 11±2.8 %, see also **Figure 3-4B**) Interestingly, treatment with DETC significantly reduced endothelium-dependent contractions in the CD and HFD group (**Figure 3-19A,B**) but not in the HFD/CD group (**Figure 3-19C**).

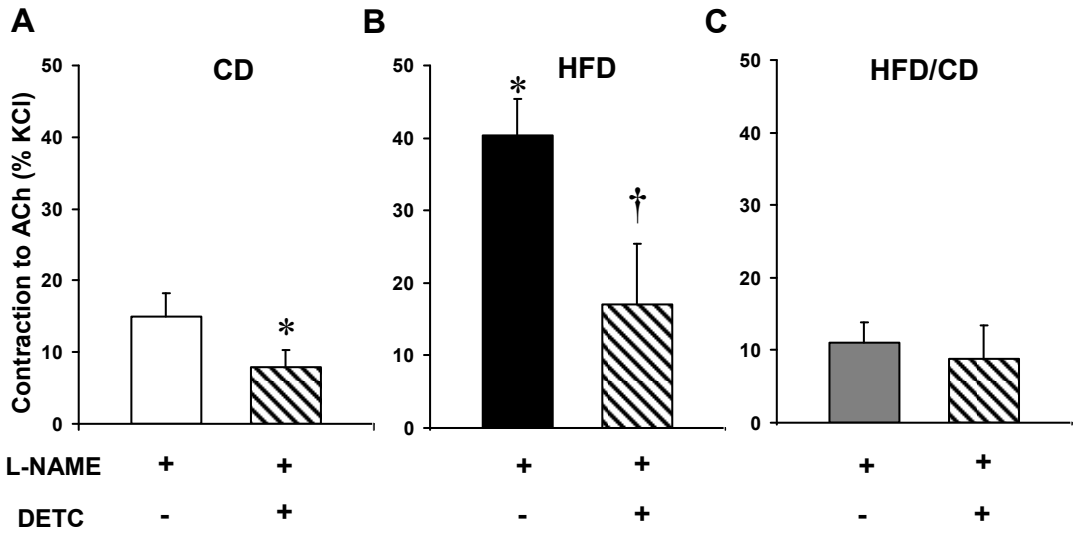

Figure 3-19 Effect of DETC on endothelium-dependent contractions in thoracic aorta of CD, HFD and HFD/CD group
Before pre-contraction with phenylephrine, rings were incubated only with L-NAME (300 µM) (CD, open bar; HFD, black bar; HFD/CD, grey bar) or with L-NAME and DETC (1 µM) (striped bars) for 30 minutes. Then, rings were exposed to cumulative concentrations of acetylcholine (ACh). Endothelium-dependent contractions at a concentration of 100 µM are shown in all three groups **(A-C)**. n=8-18 animals/group. Data are shown as a percentage of KCl-induced contraction. Data represent means±SEM.* $p<0.05$ vs. rings treated with L-NAME in the CD group, † $p<0.05$ vs. rings treated with L-NAME in the HFD group.

3.8 Vascular reactivity in wild type and Cyp2E1-deficient mice

To test whether Cyp2E1 plays a role in vascular reactivity, wild type and Cyp2E1-deficient mice were analyzed. Aortic rings were treated with different vasoactive substances to test contractile (phenylephrine, 5-hydroxytryptamine, acetylcholine) and relaxant (acetylcholine) responses. Additionally, these mice were used to verify the results obtained with the Cyp2E1-inhibitor, DETC, and additionally to test its specificity for Cyp2E1 in this experimental setting.

3.8.1 Responses to vasoconstrictors phenylephrine and 5-hydroxytryptamine

Phenylephrine-and 5-hydroxytryptamine-induced contractions were investigated in aorta of wild type (WT) and Cyp2E1-deficient (Cyp2E1$^{-/-}$) mice. Contractions induced by phenylephrine at a concentration of 1 µM were similar in WT and Cyp2E1$^{-/-}$ mice (WT: 54.1±6.3 % vs. Cyp2E1$^{-/-}$: 57.3±2.6 %) (**Figure 3-20A**). However, 5-hydroxytryptamine-induced contractions at a concentration of 10 µM were significantly decreased in Cyp2E1$^{-/-}$ mice (WT: 88.3±4.9 % vs. Cyp2E1$^{-/-}$: 64.7±4.7 %) (**Figure 3-20B**).

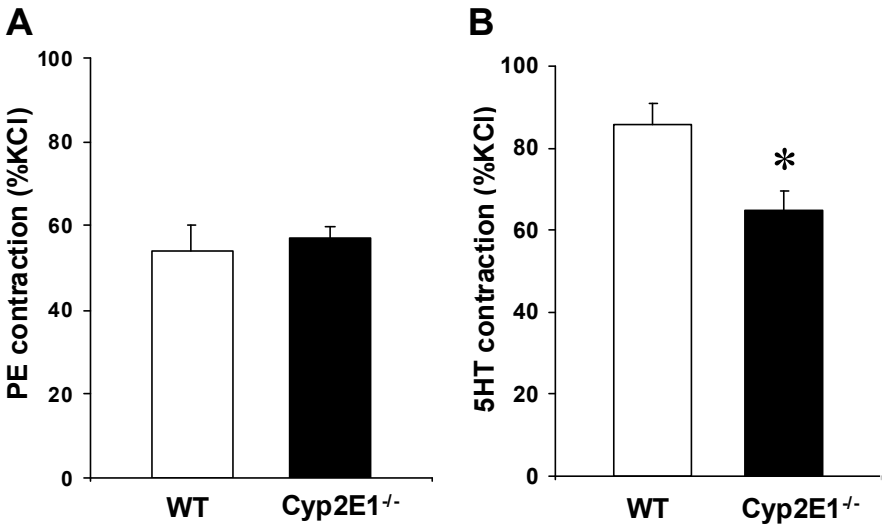

Figure 3-20 Contractile responses to phenylephrine and 5-hydroxytryptamine in wild type and Cyp2E1-deficient mice
Phenylephrine- (PE, 1 µM) **(A)** and 5-hydroxytryptamine (5-HT, 10 µM)-induced contractions **(B)** in wild type (WT, open bars) and Cyp2E1-deficient (Cyp2E1$^{-/-}$, black bars) mice are represented. n=10 WT animals; n= 17 Cyp2E1$^{-/-}$ animals. Data are shown as a percentage of KCl-induced contraction. Data represent means±SEM.* $p<0.05$ vs. WT.

3.8.2 Endothelium-dependent relaxation

In aortic rings of WT and Cyp2E1$^{-/-}$ mice endothelium-dependent relaxation were analyzed using acetylcholine. Maximal acetylcholine-induced relaxation was impaired in Cyp2E1$^{-/-}$ mice compared to WT mice (WT: -73.4±5.8 %; Cyp2E1$^{-/-}$: -53.4±7.4 %) (**Figure 3-21**). Inhibition of NO with L-NAME blocked endothelium-dependent relaxation completely in WT and Cyp2E1$^{-/-}$ mice (data not shown).

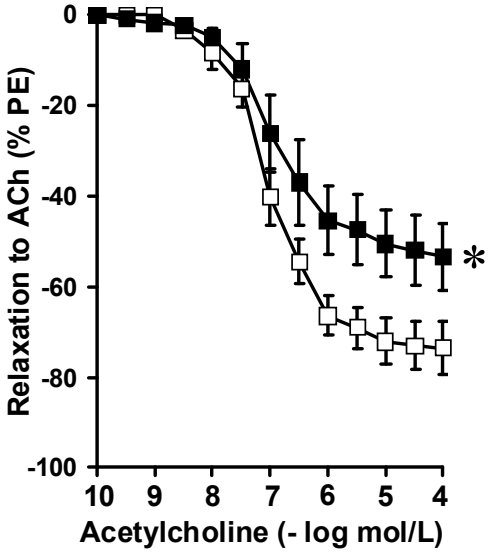

Figure 3-21 Acetylcholine-relaxation in wild type and Cyp2E1-deficient mice
To test endothelium-dependent relaxation in aortic rings of experimental mice, vessels were pre-contracted with phenyleprhine to a 50 % contraction to KCl followed by cumulative concentration of acetylcholine (ACh, 0.1 nM-100 µM). WT (open squares); Cyp2E1$^{-/-}$ (black squares) mice. n= 5 WT animals; n= 6 Cyp2E1$^{-/-}$ animals. Data are shown as a percentage of phenylephrine (% PE)-induced pre-contraction. Data represent means±SEM.* $p<0.05$ vs. WT.

3.8.3 Acetylcholine-induced endothelium-dependent contractions

WT and Cyp2E1$^{-/-}$ mice were used to test whether Cyp2E1 plays a role in acetylcholine-induced endothelium-dependent contractions. Endothelium-dependent contractions induced by acetylcholine were increased by 2 fold in Cyp2E1$^{-/-}$ mice compared to WT mice (WT: 7.3±3.7 %; Cyp2E1$^{-/-}$: 14.7±3.2 %) (**Table 3-4**). Additionally, it was tested whether the increase in acetylcholine-induced contractions in Cyp2E1$^{-/-}$ mice is initiated by COX-dependent release of prostanoids. Therefore, vessels were either pre-incubated with L-NAME (300 µM) alone or L-NAME + meclofenamate (1 µM) , a non-selective competitive inhibitor of COX-1 and COX-2 (Kretz *et al.*, 2006) for 30 minutes, pre-contracted with phenylephrine and exposed to acetylcholine at a high concentration of 100 µM. Interestingly, acetylcholine-induced contractions in Cyp2E1$^{-/-}$ mice were abolished by addition of meclofenamate (**Table 3-4**).

Inhibitors	L-NAME	L-NAME + Meclofenamte
WT	7.3±3.7	NA
Cyp2E1$^{-/-}$	14.7±3.2 *	0±0 †

Table 3-4 Endothelium-dependent contractions induced by acetylcholine in wild type and Cyp2E1-deficient mice
Contractions to acetylcholine at a concentration of 100 µM in aortic rings of WT and Cyp2E1$^{-/-}$ mice are represented. Vessel rings of WT mice were only incubated with 300 µM of L-NAME while rings of Cyp2E1$^{-/-}$ mice were incubated with L-NAME or L-NAME+meclofenamate (1 µM) for 30 minutes before pre-contraction with cumulative concentrations of phenylephrine (0.1 nM-1µM). Endothelium-dependent contractions were detected at a concentration of 100 µM of acetylcholine. n= 6 WT animals; n=6-12 Cyp2E1$^{-/-}$ animals. Data are shown as a percentage of KCl (% KCl)-induced contraction. Data represent means±SEM.* $p<0.05$ vs. WT, † $p<0.05$ vs. Cyp2E1$^{-/-}$ L-NAME. NA, not analyzed.

3.9 Effect of DETC on vascular reactivity in wild type and Cyp2E1-deficient mice

3.9.1 Impact of DETC on phenylephrine- and 5-hydroxytryptamine-mediated contractions

Aortic rings from WT and Cyp2E1$^{-/-}$ mice were incubated with no inhibitor (Ø) or different concentrations of DETC, (100 µM or 500 µM), followed by phenylephrine treatment. A concentration of 100 µM of DETC increased contraction to phenylephrine (1 µM) in WT (54.1±6.3 % vs. 74±11 %) and Cyp2E1$^{-/-}$ mice (56.5±2.5 % vs. 73.2±8.2 %), (**Table 3-5A**) but not 5-hydroxytryptamine-induced contractions (WT: 88.3±4.9 % vs. 91.5±6.1 %; Cyp2E1$^{-/-}$: 67.4±5.2 % vs. 63.7±7.5 %) (**Table 3-5B**). A concentration of 500 µM further enhanced phenylephrine-induced contractions (1 µM) in WT (54.1±6.3 % vs. 96±3.8 %) and Cyp2E1$^{-/-}$ mice (56.5±2.5 % vs.105±12.1 %) (**Table 3-5A**). Contractions to 5-hydroxytryptamine were decreased with DETC at a concentration of 500 µM only in WT (88.3±4.9 % vs. 74.6±4.7 %) but not in Cyp2E1$^{-/-}$ mice (67.4±5.2 % vs. 62.6 ±10.1 %) (**Table 3-5B**).

A

Inhibitors	Ø	DETC 100 µM	DETC 500 µM
WT	54.1±6.3%	74±11%*	96±3.8% *
Cyp2E1$^{-/-}$	56.5±2.5%	73.2±8.2%†	105.2±12.1%†

B

Inhibitors	Ø	DETC 100 µM	DETC 500 µM
WT	88.3±4.9%	91.5±6.1%	74.6±4.7%*
Cyp2E1$^{-/-}$	67.4±5.2%	63.7±7.5%	62.6±10.1%

Table 3-5 Different DETC concentrations and their effect on phenylephrine- and 5 –hydroxytryptamine- induced contractions in wild type and Cyp2E1-deficient mice
Table shows phenylephrine- **(A)** and 5-hydroxytryptamine-induced contractions **(B)** at a concentration of 1 µM and 10 µM in aortic rings, respectively, in WT and Cyp2E1$^{-/-}$ mice without inhibitor (Ø) or pre-incubated for 30 minutes with either 100 µM or 500 µM of DETC. n= 7-17 WT animals; n=8-12 Cyp2E1$^{-/-}$ animals. Data are shown as a percentage of KCl (% KCl)-induced contraction. Data represent means±SEM.* $p<0.05$ vs WT, † $p<0.05$ vs Cyp2E1$^{-/-}$.

3.9.2 Effect of DETC on endothelium-dependent contractions induced by acetylcholine

Acetylcholine-induced contractions at a high concentration (100 µM) were investigated in NO-depleted condition without or with DETC (100 µM) in WT and Cyp2E1$^{-/-}$ mice. **Table 3-6** represents that endothelium-dependent contractions induced by acetylcholine are decreased by DETC in WT (7.3±3.7 % vs. 2.1±1.4), but not in Cyp2E1$^{-/-}$ mice (14.9± 3.2 % vs. 13.3±3.4 %).

Inhibitors	L-NAME	L-NAME + DETC
WT	7.3±3.7%	2.1±1.4% *
Cyp2E1$^{-/-}$	14.9±3.2%	13.3±3.4%

Table 3-6 Effect of DETC on acetylcholine-induced contractions in wild type and Cyp2E1-deficient mice
Represented are contractions induced by acetylcholine at a concentration of 100 µM in the presence of L-NAME (300 µM) or L-NAME+DETC (100 µM) in aortic rings from WT and Cyp2E1$^{-/-}$ mice**.** n= 6 WT animals; n= 6-12 Cyp2E1$^{-/-}$ animals. Data are shown as a percentage of KCl (% KCl)-induced contraction. Data represent means±SEM.* $p<0.05$ vs. WT L-NAME.

4 Discussion

The main findings of the current study are as follows: (I) HFD causes heterogenous changes in vascular reactivity, increases body weight and leads to impaired glucose tolerance; (II) a diet switch from HFD to CD induces selective changes in vascular reactivity despite normal body weight and glucose tolerance; (III) transcriptomic analysis reveals 46 genes in which at least 2 exons show a significant expressional change in any of the treatment groups; (IV) identification of *Dpp10*, *Car 3*, *Cidec* and *Cyp2E1* being differentially expressed by HFD and HFD/CD in aortic tissue; (V) characterization of aortic Cyp2E1 splice variants; (VI) Cyp2E1 plays a role in regulation of vascular function.

4.1 Effect of HFD and HFD/CD on body weight and glucose tolerance

Obesity is characterized by an imbalance between energy intake and energy expenditure, in which energy intake is increased and/or energy expenditure is decreased. This leads to an increase in body weight which can be mainly attributed to more body fat or adipose tissue. Adipose tissue is composed of adipocytes. Obesity increases number (hyperplasia) and size (hypertrophy) of adipocytes, the predominant cell type in adipose tissue (Jequier, 2002). Systemically, obesity is associated with various alterations including impaired glucose tolerance and insulin resistance in humans (Kahn *et al.*, 2006; Karam & McFarlane, 2011) and animals (Woods *et al.*, 2004; Mundy *et al.*, 2007b). After reduction of body weight glucose tolerance improves (Colman *et al.*, 1995).

The C57BL/6J mouse strain is suitable to study diet-induced obesity (Surwit *et al.*, 1988; Collins *et al.*, 2004) because these mice develop obesity and diabetes upon high-fat diet feeding resembling human obesity and diabetes (Buettner *et al.*, 2007). Many factors such as composition of fatty acids, secretion of signaling molecules from adipocytes, stress, low satiating effects of fat, texture and odour of the high-fat diet, frequency and rate of food intake are described in rodents leading to an increase in body weight (Hariri & Thibault, 2010). In rodents usually high-fat diets containing 30-78 % kcal from fat are used to induce obesity (Buettner *et al.*, 2007) compared to normal chow diet containing 3.5-12 % kcal from fat (Reliene & Schiestl, 2006; Gajda *et al.*, 2007). In the present study, the high-fat/high-carbohydrate diet used from Research Diets (D12079B) contains 41 % kcal from fat, 45 % kcal from carbohydrates and 17 % kcal from protein. This diet induced a significant increase in body weight after 30 weeks in the HFD group. In accordance to the present data, studies showed that high-fat/high-carbohydrate diets increase body weight in C57BL/6J mice (Surwit *et al.*, 1988; Klaus, 2005). At 30 weeks of diet, the mean value of total body weight reached in mice of the HFD group was 37 grams. This was 6 grams more compared to CD and HFD/CD.

Another study showed a 14 grams increase in body weight in C57BL/6J mice after 30 weeks, but the high-fat diet used contained 60 % kcal from fat (Hoffler *et al.*, 2009). The mice used in this study were held in a specific-pathogen free environment in cages with separate air condition. As germ-free C57BL/6J mice increase less in body weight compared to conventionally housed mice (Rabot *et al.*, 2010) this might be a possible explanation for the overall lower weight gain in mice of the present study. Furthermore, body weight in diet-induced obesity mice is not as high as in genetically-modified mice (Woods *et al.*, 2004; Murphy *et al.*, 2010). Surprisingly, body weight of mice in the different treatment groups was similarly increased after 15 weeks. Another study showed that the same high-fat diet (D12079B) induces a significant increase in body weight gain of 6 grams in C57BL/6J mice after 15 weeks of high-fat diet compared to control diet (Mundy *et al.*, 2007b). Ito *et al.* even showed that C57BL/6J mice fed for 15 weeks with the high-fat diet (D12079B) had an increased body weight gain of 16 grams (Ito *et al.*, 2006). It suggests that in the present experimental set-up (i) weight gain is highly variable in the first 15 weeks of age and that (ii) body weight gain is independent of dietary fat content for the first 15 weeks of diet. It is possible that body weight in all treatment groups was similar after 15 weeks because mice were only 4 weeks old when the dietary treatment started. The energy from the first 15 weeks of HFD feeding was exclusively used for growth of the animals. Important to note is also that total weight gain in all dietary treatment groups was highest after 15 weeks (approximately 15 grams of weight gain). Mice in the HFD group increased their body weight after 30 weeks to 6 grams whereas in the CD and HFD/CD group an increase of only 2 grams was observed. It suggests that longer feeding of this diet is relevant for HFD-dependent increase in body weight. Therefore it was not surprising that total weight gain in the HFD/CD group was lower compared to the HFD group and similar to the CD group. The control diet used in the CD and HFD/CD group contained 12 % kcal from fat, 65 % kcal from carbohydrates, and 22 % kcal from proteins. Studies with animals showed that diets low in fat can induce weight loss after 16 weeks of feeding a high-fat diet and switching to a low-fat diet for 16 weeks (Parekh *et al.*, 1998). Other studies showed that C57BL/6J mice develop hyperplastic and hypertrophic fat cells upon HFD feeding (Surwit *et al.*, 1995). Hill and colleagues reported that hyperplasia of fat cells induced by 17 weeks of high-fat diet intake was reversed after diet was switched to low fat diet. Interestingly, if the diet switch was only carried out after 30 weeks hyperplasia of fat cells was not reversible (Hill *et al.*, 1989).

Not only the amount of energy from fat is important to develop obesity but rather the composition of fatty acids is crucial (Storlien *et al.*, 2001; Moussavi *et al.*, 2008). Fat in the high-fat diet (D12079B) from Research Diets derives mainly from milk fat. Usually, 70 % of fatty acids in milk fat are saturated, 25 % are monounsaturated, and approximately 2 % are polyunsaturated (Mansson, 2008). Nutrition rich in saturated fatty acids (SFAs) increase the risk to develop obesity in humans (van Marken Lichtenbelt *et al.*, 1997). Wang *et al.* showed that a diet high in SFAs also increases

body weight in C57BL/6J mice (Wang *et al.*, 2002). SFAs are usually not used for energy expenditure, but stored as triglycerides in adipose tissue. However, polyunsaturated and monounsaturated fatty acids are used for energy expenditure and stored less in adipose tissue (Storlien *et al.*, 2001). It is also known that SFAs from animal fat mainly contain long-chain fatty acids compared to plant fat which contains medium-chain fatty acids and that this is related to the development of obesity (Hariri & Thibault, 2010). The high content of SFAs in the diet may contribute to the higher weight gain in mice fed with HFD in the present study.

High dietary fat intake leads to impaired glucose tolerance and reduces the effect of insulin to lower glucose levels in the blood (Lichtenstein & Schwab, 2000). A study performed by Takahashi and coworkers showed that a higher percentage of fat in a high-fat diet is correlated with glucose intolerance (Takahashi *et al.*, 1999). The dietary treatment used in this study not only had an effect on body weight but also had an impact on glucose tolerance in these mice. A GTT performed with mice fed for 30 weeks with HFD showed that glucose tolerance in these mice was impaired after 2 hours but not in HFD/CD group. Glucose concentration in the blood of mice in the HFD/CD group was reduced after 2 hours compared to the HFD group and similar to the CD group. Parekh et al. showed that plasma glucose and insulin levels (analyzed with a Beckman Glucose Analyzer 2 (Taylor & Pennock, 1981) and double antibody rat insulin radioimmunoassay kit, respectively) were decreased after switching from 16 weeks of high-fat diet feeding to a low fat diet for 16 weeks (Parekh *et al.*, 1998). The reduced weight gain in the HFD/CD group compared to the HFD group might be the reason why glucose tolerance is normal in these mice. Similarly, weight loss in severely obese humans by bariatric surgery and dietary intervention by reduction in dietary fat intake improves glucose tolerance (Long *et al.*, 1994; Fruchter, 2001).

This study has some limitations in regard to phenotypic and metabolic analysis: Daily food intake was not measured in these experimental groups. An irregular food intake might explain why animals had similar weights after 15 weeks and the mean total body weight of mice in the HFD group was only 37 grams. Furthermore, fat pads (subcutaneous and epididymal) of these animals were not weighed which would have indicated whether adipose tissue amount is different between the dietary treatment groups. It would have been also important to measure plasma lipids such as cholesterol (Do *et al.*, 2011) and common adipokines or cytokines such as leptin (Ahren *et al.*, 1997) and TNFα (Mito *et al.*, 2000) which are secreted by adipose tissue. Leptin, for example, is an important adipokine regulating food intake and body weight (Zhang *et al.*, 1994). It is elevated in obese humans (Saad *et al.*, 1998) and also in mice (Frederich *et al.*, 1995). This in fact indicates that high levels of leptin cause leptin resistance. Dysfunction of leptin receptors in the hypothalamus lead to uncontrolled food intake and behavior (Frederich *et al.*, 1995). In addition to the GTT, an insulin tolerance test should have been performed to test whether secretion of insulin and its action on lowering blood glucose is affected, especially in the HFD group. Furthermore,

pancreatic β-cells, skeletal muscle, liver and adipocytes play an important role in regulating glucose metabolism. Additional experiments using these cells and organs would be needed to explain why glucose tolerance is impaired by HFD and normal after HFD/CD. It is known that pancreatic β-cells synthesize and secrete insulin to maintain blood glucose level homeostasis. In obese humans, a dysfunction of pancreatic β-cells can lead to a diminished insulin secretion. The cells are not able anymore to release insulin quickly upon high glucose levels in the blood (Kahn, 2001). In this study, impaired glucose tolerance after HFD might be explained in part by damage in β-cell function not able to release insulin. Mundy and colleagues showed that an impaired glucose tolerance was already present after 15 weeks of feeding with high-fat diet (D12079B) (Mundy *et al.*, 2007b). This implicates that a disturbance in glucose tolerance already occurs after 15 weeks of high-fat diet treatment and sustains after longer feeding of 30 weeks. The data of the present study show that a reduction in dietary fat content after 15 weeks can normalize an impaired glucose tolerance.

4.2 Influence of HFD and HFD/CD on vascular reactivity

An intact endothelial function is important to maintain healthy vascular homeostasis (Caballero, 2003). Regulation of vascular tone by release of vasoactive molecules such as vasoconstrictors and vasodilators is one of the main functions of the endothelium. Obesity leads to an imbalance between secretion of vasoconstrictors and vasodilators, favoring the release of vasocontractile molecules but also of inflammatory and growth-promoting molecules. A consequence can be endothelial dysfunction and development of vascular diseases (Quyyumi, 1998; Caballero, 2003). Weight loss improves endothelial dysfunction (Caballero, 2003) but other studies also showed that if endothelial function is once damaged, for example by obesity, it is not possible to completely restore its function even after normalization of body weight (Brook *et al.*, 2004). A possibility to assess functional or dysfunctional endothelium is measurement of vascular reactivity.

4.2.1 Phenylephrine and 5-hydroxytryptamine-induced contractions

Phenylephrine causes vasoconstriction in isolated thoracic aorta of C57BL/6J mice (Russell & Watts, 2000), mainly by direct binding to the G-protein coupled α_1-adrenergic receptor on VSMCs (Piascik *et al.*, 1996). HFD feeding in mice increases vascular contractions to phenylephrine (Molnar *et al.*, 2005). In the present study, treatment with HFD and HFD/CD for 30 weeks increased phenylephrine-mediated contractions in thoracic aorta of mice. The increased response to HFD could be due to an increased expression of α_1-adrenergic receptors and phenylephrine-induced downstream signalling stimulated by HFD. Additionally, expression and release of dilatory molecules might be decreased which increases phenylephrine-mediated contractions. In the present study, inhibition with L-NAME showed that basal NO

availability is reduced by HFD and HFD/CD in comparison to CD (data not shown). This is also shown by other studies (Bourgoin *et al.*, 2008). Smith *et al*. showed that body weight is increased, glucose tolerance is impaired and phenylephrine-mediated contraction to phenylephrine is enhanced in aorta of rats fed a high-fat diet (35 % from fat) for 10 weeks (Smith *et al.*, 2006). Increased contractile responses to the vasoconstrictor phenylephrine could be also explained by the increased body weight and the impaired glucose tolerance in mice of the present study. It might be possible that HFD affected phenylephrine-mediated contractions by an increased number of α_1-adrenergic receptors on VSMCs and promoting an increase in contractile responses. A study performed by Hodgkin *et al*. showed that total number of α_1-adrenergic receptors in aorta of rats is increased due to dietary fat treatment (Hodgkin *et al.*, 1991). The increased phenylephrine-mediated contractile response in the HFD/CD group could be due to a memory effect of VSMCs. Increased expression of α_1-adrenergic receptors probably cannot be reversed after reducing the fat content in the diet after 15 weeks even though body weight and glucose tolerance are similar to CD. This might also explain that changes in vasoconstriction responses already occur early possibly even after 15 weeks of HFD feeding. Furthermore, these data suggest that a switch to CD after 15 weeks of HFD only normalizes body weight and glucose metabolism which is partly independent from changes in the vasculature. Surprisingly, contractile responses to 5-hydroxytryptamine were not affected by HFD even though it has been shown that diet-induced obesity augments 5-hydroxytryptamine-induced contraction in isolated mesenteric arteries (Boustany-Kari *et al.*, 2007) and aorta of rats (Ghatta & Ramarao, 2004). It is possible that smaller vessels such as mesenteric arteries are more prone to increased vasoconstriction than aorta. However, Ghatta *et al.* showed that contractile responses to 5-hydroxytryptamine were higher in aortic rings from rats fed a high-fat diet containing 58 % from fat suggesting species-specific effects (Ghatta & Ramarao, 2004). 5-hydroxytryptamine-induced contractions are mainly mediated by activation of S_2-receptors on VSMCs (Vanhoutte *et al.*, 1984). Expression of these receptors might not be changed by HFD. On the other hand, changes in adrenergic receptor expression might be more susceptible and sensitive to HFD than serotonergic receptors. Important to note is that contractions to 5-hydroxytryptamine are higher compared to phenylephrine-induced contractions in the CD group. It is possible that under normal conditions the number of expressed S_2-receptors is higher compared to α_1-adrenergic receptor on VSMCs. HFD is not able to increase expression of S_2- receptors because of saturation which might not be the case for α_1-adrenergic receptors. Interestingly, HFD did not increase 5-hydroxytryptamine-contractions, but a slight and significant increase in these contractions was observed with HFD/CD. Based on the results of the HFD feeding it could be that not HFD feeding but a switch to CD feeding is responsible for the increase in 5-hydroxytryptamine contractions. Overall, 5-hydroxytryptamine-induced contractions are more resistant to dietary interventions independent of changes in body weight and glucose metabolism than contractile responses to phenylephrine.

Taken together, HFD and HFD/CD increases contractions to phenylephrine strongly, while contractions to 5-hydroxytryptamine were only slightly increased after HFD/CD diet.

4.2.2 Acetylcholine-induced relaxation and contraction

Acetylcholine causes endothelium-dependent relaxation in isolated thoracic aorta of C57BL/6J mice (Russell & Watts, 2000) by the release of NO (Feletou *et al.*, 2008). In the present study, endothelium-dependent relaxation in aorta induced by acetylcholine was unaffected by HFD. Unchanged vascular relaxation by dietary interventions indicates that eNOS activity and NO availability are intact. Other researchers showed that HFD feeding for 15 weeks using the same type of diet (D12079B from Research Diets) also did not impair endothelium-dependent relaxation (Mundy *et al.*, 2007b). Similarly, feeding of C57BL/6J mice with a HFD from Harlan Teklan, containing 42 % kcal from milk fat for 26 and 30 weeks showed that acetylcholine-induced endothelium-dependent relaxation in isolated aorta and mesenteric arteries was normal (d'Uscio *et al.*, 2001; d'Uscio *et al.*, 2002). The results of this study together with findings from the above mentioned publications indicate that (i) neither short-term nor long-term feeding of HFD alters endothelium-dependent relaxations in different vascular beds which could be due to normal NO availability. Despite normal dilatory response induced by acetylcholine after HFD the source of relaxant molecules can change: Obesity can activate expression of inflammatory genes such as iNOS (Noronha *et al.*, 2005). iNOS releases up to 1000-fold more NO than eNOS (Nathan, 1997). Noronha *et al*. showed that diet-induced obesity in C57BL/6J mice increases expression of iNOS in aorta raising NO production (Noronha *et al.*, 2005). It suggests that endothelium-dependent relaxation in the CD group is mediated by NO release from eNOS while relaxation in the HFD groups might be mediated by NO produced by increased expression of iNOS.

On the other hand, it is known that ECs generate high amounts of ROS during obesity (Traupe *et al.*, 2002; Taniyama & Griendling, 2003; Mundy *et al.*, 2007b). Uncoupling of eNOS forms O_2^- and NO. This leads to scavenging of NO by O_2^- resulting in $ONOO^-$ formation which decreases NO availability and NO-mediated relaxation (Vanhoutte *et al.*, 2009). Additionally, O_2^- is converted by SOD to H_2O_2 which causes relaxation (Shimokawa, 2010). In the present study, it is possible that HFD feeding increases formation of O_2^- leading to H_2O_2 which would explain the normal relaxation. Additionally, H_2O_2 can be further converted by catalase into H_2O and O_2 or to •OH (Taniyama & Griendling, 2003). Interestingly, •OH enhances endothelium-dependent relaxation in mice fed a high-fat diet (Mundy *et al.*, 2007a). To note is that acetylcholine can also stimulate the production of ROS (Yao *et al.*, 1999; Oldenburg *et al.*, 2002; Noronha *et al.*, 2005). These observations indicate that acetylcholine-induced endothelium-dependent relaxation during HFD is mediated by the release of ROS. In rats, high-fat diet feeding decreases acetylcholine-induced

relaxation after a feeding period of 28 weeks (Roberts *et al.*, 2005). It is possible that impact of high-fat diet feeding on endothelium-dependent relaxation varies between species. On the other hand, Ketonen *et al.* showed that endothelium-dependent relaxation is impaired in aorta of C57BL/6J mice after 20 weeks of high-fat diet (Ketonen *et al.*, 2010). These authors showed that decreased endothelium-dependent relaxation is associated with dysregulation of eNOS phosphorylation and eNOS uncoupling because of increased superoxide production (Ketonen *et al.*, 2010). The impairment in vascular relaxation observed by Ketonen *et al.* might be due to higher fat content (61 % kcal from fat) in the diet used. In this study, endothelium-dependent relaxation was unaffected by HFD/CD. Ketonen *et al.* showed that caloric restriction after high-fat diet feeding normalizes endothelium-dependent relaxation because of reduced release of reactive oxygen species (Ketonen *et al.*, 2010). Endothelium-dependent relaxation in the HFD/CD group probably occurs via reduced release of reactive oxygen species or by NO either generated by iNOS or eNOS. Furthermore, 15 weeks of feeding with the high-fat diet (D12079B) has no impact on vascular relaxation as shown by Mundy *et al*. (Mundy *et al.*, 2007b). Therefore, it was not surprising that endothelium-dependent relaxation in the HFD/CD group was similar to the CD group. The data of the present study also show that relaxant responses to dietary interventions are more resistant to changes compared to vasoconstrictor responses mediated by phenylephrine.

Inhibition of NO-synthase blocks the release of NO in isolated arteries (Vanhoutte & Tang, 2008). This evokes endothelium-dependent contractions by conversion of AA by COX-1 to endoperoxides and prostaglandins which bind to TP receptors on VSMCs to mediate contractions (Feletou *et al.*, 2008; Vanhoutte & Tang, 2008). In the present study, acetylcholine-induced endothelium-dependent contractions were increased by HFD but not by HFD/CD. An increase in endothelium-dependent contractions by HFD might involve an increased COX-1 activity and enhanced release of endoperoxides and prostaglandins. Additionally, expression of TP-receptors is enhanced due to increased release of endoperoxides and prostanoids. Interestingly, Mundy *et al*. showed that HFD feeding in C57BL/67 mice for 15 weeks did not significantly increase endothelium-dependent contractions upon NO-synthase inhibition with L-NAME (Mundy *et al.*, 2007b). This indicates that a longer duration of HFD feeding facilitates endothelium-dependent contractions. Furthermore, generation of ROS by HFD promote an increase in endothelium-dependent contractions in mice which is related to increased expression of TP receptors (Gollasch, 2002; Traupe *et al.*, 2002). Specifically, O_2^- can scavange NO leading to $ONOO^-$ favoring endothelium-dependent contractions (Vanhoutte *et al.*, 2009). HFD fed animals of this study showed increased body weight and glucose tolerance. Increased body weight leads to endothelium-dependent contractions in aorta of mice fed a HFD for 30 weeks (Traupe *et al.*, 2002). Due to the literature high glucose levels and obesity facilitate an increase in ROS availability in the vasculature, especially O_2^- and increase the expression of COX-1 which leads to endothelium-dependent

contraction (Vanhoutte *et al.*, 2009). Furthermore, O_2^- stimulates COX-1 metabolizing AA to endoperoxides and prostaglandins (Vanhoutte, 2002). Endothelium-dependent-contractions in the HFD group might be due to increased release of O_2^-. Endothelium-dependent contractions in the HFD/CD group were similar to those observed with CD. This might be due to the beneficial effect of the diet switch, reduced production of O_2^-, a normalized COX-1 activity and reduced release of endoperoxides and prostaglandins.

Only HFD increased endothelium-dependent contractions possibly by increased release of O_2^- while endothelium-dependent-relaxation remained unaffected by HFD and HFD/CD.

4.3 Vascular gene expression profiling after different dietary interventions

Since 1995 DNA microarray technology is a powerful tool to identify differences in gene expression patterns (Schena *et al.*, 1995), and is successfully used to determine obesity-associated changes in gene expression (Mzhavia *et al.*, 2008; Joo & Yun, 2011). Many studies have shown that high-fat diet and obesity affect gene expression in adipose tissue (Urs *et al.*, 2004; Baranova *et al.*, 2005; Rodriguez-Acebes *et al.*, 2010; Ichioka *et al.*, 2011), liver (Kim *et al.*, 2004), and skelatal muscle (Sparks *et al.*, 2005). Soukas and colleagues were the first performing DNA microarray experiments related to obesity in 2000 (Soukas *et al.*, 2000). The effect and improvements of weight loss due to caloric restriction on gene expression was mainly investigated in adipose tissue in rodents and humans using transcriptomics tools (Viguerie *et al.*, 2005). Only few studies exist in which the effect of high-fat diet feeding on gene expression profiling in aorta is explored (Mzhavia *et al.*, 2008; Dejeans *et al.*, 2009; Mak *et al.*, 2010; Rull *et al.*, 2010). The impact of dietary fat restriction on vascular gene expression is not yet investigated. Furthermore, most of the studies mentioned here used 3`based gene expression arrays. Since 2005, Affymetrix offers exon microarrays (Affymetrix., 2005b) for humans, mouse and rats which allow implementation of gene expression analysis of the whole gene and the identification of alternatively spliced genes (Whistler *et al.*, 2010; Paloschi *et al.*, 2011). This is possible because probes are located at each exon over the entire length of the transcript (detailed description of the Affymetrix exon arrays is described in Materials and Methods **2.2.4**) This is different to the 3`based gene expression arrays in which the probes only target the 3`end of the gene. The Affymetrix exon array was successfully used in various disease-related studies to identify gene expression changes and splice variants (Gardina *et al.*, 2006; Thorsen *et al.*, 2008; Bemmo *et al.*, 2010). Therefore, in the present study, Affymetrix DNA exon microarray was chosen to assess gene expression changes by dietary treatments but also exon expression changes to distinguish between different isoforms of a gene. Gene expression analysis revealed that dietary treatments with HFD and HFD/CD only

affected the expression of few genes in aortic tissue with a total of 37 genes. Exon expression analysis identified 46 genes in which at least 2 exons of a gene showing to be differentially expressed. In addition, only small expressional changes with a maximal $\log_2$ fold change of 2.2 were identified. This indicates that long-term dietary treatment affects only few genes in aortic tissue with small expressional changes. Dejeans and colleagues performed a DNA microarray experiment with aortic tissue from C57BL/6J mice fed a high-fat diet for only 3 weeks. Surprisingly, these researchers showed that 448 genes were differentially expressed upon high-fat diet treatment (Dejeans *et al.*, 2009). This might indicate that a short-term dietary treatment of 3 weeks affects expression of more genes than a long-term treatment of 30 weeks. Interestingly, the expressional changes were also not very high (maximal $\log_2$ fold change of 4, but most genes showed a $\log_2$ fold change of 0.5-2.5) (Dejeans *et al.*, 2009) which is in line with our findings. An additional study by Mzhavia *et al.* performed a microarray of aortic tissue from C57BL/6J mice which were fed with a high-fat diet containing 35 % from fat for 16-20 weeks identified Cyp2E1 as one of the significant differentially expressed genes (Mzhavia *et al.*, 2008) which is in line with findings of this study.

Gene Ontology analysis revealed that identified genes are associated with processes due to cytoskeleton and extracellular matrix alterations (Dejeans *et al.*, 2009) whereas in the present study identified genes were assigned to processes involved in regulation of vasoconstriction, circulatory system process, blood circulation, and positive regulation of vasodilatation. It indicates that short-term dietary treatment affects genes which are important for vessel architecture and structure whereas a long-term dietary treatment has an impact on vessel function. An artery mainly contains ECs and VSMCs (Pugsley & Tabrizchi, 2000). Both cell types are important to modulate vascular function. Using ECs (Beyer *et al.*, 2008) or VSMCs as starting material for array analysis would have probably led to different results. A separate microarray analysis of ECs and VSMCs would avoid nivellation problems which means that a particular gene is not identified in a whole aortic tissue approach if the gene is upregulated in ECs and downregulated in VSMCs. Another possibility could be that the observed changes in vascular reactivity due to HFD and HFD/CD are not exclusively mediated by expressional changes of genes in aortic tissue. Expressional changes of genes in liver, skeletal muscle and adipose tissue are evenly important in modulating changes in vascular reactivity. Gene expression of peroxisome proliferator-activated receptors (PPARs) in liver of mice was shown to be affected by long-term high-fat diet treatment of 12 weeks (Kim *et al.*, 2004). PPAR-γ was shown to be involved in modulation of angiotensin II and thromboxane A_2 -mediated renal vasoconstriction in rats (Newaz *et al.*, 2006). Furthermore, high-fat diet treatment in mice reduces expression of genes involved in oxidative phosphorylation in mitochondria of skeletal muscle (Sparks *et al.*, 2005). Oxidative phosphorylation is an important process to provide energy in form of ATP from oxygen (Madamanchi *et al.*, 2005). Under pathophysiological conditions, possibly initiated through high fat diet, oxidative phosphorylation is partially uncoupled leading

to production of O_2^-. As outlined in **1.4** ROS, and specifically O_2^- are implicated to affect vascular reactivity and leading to endothelial dysfunction (Cai & Harrison, 2000). Several adipokines such as leptin and adiponectin as well as inflammatory cytokines such as TNF-α and IL-6 released from adipocytes during obesity are known to have an impact on vascular reactivity (Kougias *et al.*, 2005; Vila & Salaices, 2005).

4.3.1 Validation and regulation of target gene expression

In order to ensure whether data obtained from DNA microarray experiments are biologically valid, data sets are usually validated using qRT-PCR and Western Blot (Chuaqui *et al.*, 2002). Often data from microarray and quantitative RT-PCR do not correlate based on biological and/or technical variations, and different analysis tools (Chuaqui *et al.*, 2002). A microarray experiment is a multi-step procedure in which it is important to keep variability (biological and technical) low in order to obtain good data. A biological variability of the sample is difficult to control. It is important to carefully prepare the sample as well as include a solid number of replicates. Technical variabilty can involve steps from isolation of RNA, reverse transcription, labeling of cDNA, hybridization of cDNA to the chip platform, and analysis of the data (Novak *et al.*, 2002; Bryant *et al.*, 2011). Therefore, care has to be taken while performing a microarray experiment to avoid irregularities which can affect the quality of the data. Sources for errors using qRT-PCR and Western Blots are amplification errors, mispriming and primer dimers, and antibodies which may affect the results negatively.

In the present study, based on statistical analysis 12 genes being differentially expressed by HFD and HFD/CD were chosen for validation with qRT-PCR. Four genes (*Dpp10*, *Car3*, *Cidec*, and *Cyp2E1*) could be validated by qRT-PCR to be differentially expressed by HFD and HFD/CD. This is in line with the results obtained by microarray analysis (see also **3.3.4**). It suggests that the microarray experiment was technically performed well because data of qRT-PCR correlate with data obtained from microarray. Furthermore, primer pair design and binding location was chosen appropriate for those genes.

Validation of target genes *Lepr* and *Dgkg* showed that both genes are only differentially expressed after HFD and not by HFD/CD as suggested by the microarray data. This indicates that the results of the qRT-PCR are not in line with the microarray data. On the other hand, it is important to note that primers designed for qRT-PCR validation only detected short sections of the sequence (max. 150 bp, mainly located in one exon or exon boundaries of the whole gene) to achieve good amplification efficiency (Wong & Medrano, 2005). This means that data obtained with qRT-PCR only could validate expression of maximal 2 exons of a gene. Further studies would be needed to find out whether expression of exons are differentially affected by dietary treatments which is suggested by line graphs (see **3.3.4**) For *Cyp2E1* primer pairs were designed for each exon of the gene to validate exon expression levels with qRT-PCR. The results showed that expression of each exon was downregulated by HFD and

HFD/CD (data not shown). The data obtained for *Lepr* and *Dgkg* also indicate that some exons of the genes are only affected by HFD and others probably by both HFD and HFD/CD. To test this primer pairs for each exon of the gene should have been designed and expression validated by qRT-PCR.

Target genes *Lyve1*, *Pdk4*, *P2rx1*, *Fkbp5*, *FMO3* and *Nrg1* were not, differentially expressed by HFD and HFD/CD as shown with qRT-PCR even though a tendency was observed. This might be due to very small differences observed and standard errors of the mean are relatively high indicating a strong variability. The results for these genes indicate that the primer pair used detects an exon which is not differentially expressed by neither HFD nor/and HFD/CD.

It is not known whether these four genes biologically interact with each other. Cyp2E1 protein levels in liver can be induced by obesity (Raucy *et al.*, 1991). Interestingly, mRNA and protein expression of aortic Cyp2E1 was increased in streptozotocin-induced diabetic rats (Schafer *et al.*, 2010). mRNA expression of *Cyp2E1* was also increased in C57BL/6J mice fed for 16-20 weeks with a high-fat diet containing 35 % from fat (Mzhavia *et al.*, 2008). In the present study, *Cyp2E1* was downregulated by HFD and HFD/CD after 30 weeks of feeding. Indeed, other researchers showed that Cyp2E1 protein expression is downregulated in interlobar arteries of spontaneous hypertensive rats at the age of 10-12 weeks. (Zhang *et al.*, 2005) This would be in line with our finding. Taken together, this indicates that expression of Cyp2E1 is affected by (i) tissue-specific regulation, (ii) various diseases, and (iii) short-term vs. long-term high-dietary feeding.

Car3 is one of 14 known carbonic anhydrase isozymes (Swenson, 2000) which was found to be expressed in low amounts in vascular smooth muscle cells of bovine aorta (Berg *et al.*, 2004). Carbonic anhydrases are usually involved in acid-base balance, ion transport, and gas exchange but it was also implicated to play a role in the regulation of vascular tone, even though concentrations are low (Berg *et al.*, 2004). Interestingly, a gene expression profiling analysis using adipose tissue from C57BL/6J mice fed a high-fat diet (21 % from fat) showed that expression of *Car3* is increased after 3 weeks of high-fat diet feeding and decreases after 5 weeks of feeding (Van Schothorst *et al.*, 2005). In the present study, HFD and HFD/CD decreased expression of *Car3* in aortic tissue after 30 weeks of dietary feeding. It might suggest that longer dietary treatment decreases expression of *Car3* in various tissues.

In the present study, *Cidec* mRNA expression levels in aortic tissue were downregulated by HFD and HFD/CD. Cidec belongs to the cell-death-inducing DFF45-like effector gene familiy and was implicated to play a role in adipocyte lipid metabolism (Magnusson *et al.*, 2008) and apoptosis (Keller *et al.*, 2008). Interestingly, Keller *et al.* showed that Cidec expression in subcutaneaous adipose tissue from obese patients (BMI $\geq$30 kg/m^2) is decreased. Another study from Magnusson and collegues reported that Cidec expression in subcutaneaous adipose tissue from obese patients (BMI of 36 kg/m^2) undergoing a very low calorie dietary treatment for 12 weeks was

decreased (Magnusson *et al.*, 2008). Together this suggests that *Cidec* expression is similar regulated in adipose and aortic tissue.

To my knowledge, nothing is known about the expression of Dpp10 in aortic tissue and impact of dietary treatments. Dpp10 belongs to the S9B prolyl oligopeptidase class of serine proteases which is highly expressed in brain and modulates voltage-gated K^+ channels (Kvs) especially Kv4-mediated A-type K^+ channels (Zagha *et al.*, 2005). Kvs are expressed in vascular smooth muscle cells (Korovkina & England, 2002) and play an important role in regulating vascular tone under normal and pathophysiological conditions (Brayden, 1996; Thorneloe *et al.*, 2001). The Kv4 subfamily is also expressed in vascular smooth muscle cells (Cox, 2005). Therefore it is not surprising that *Dpp10* was identified in aortic tissue. It is possible that downregulation of *Dpp10* expression by HFD and HFD/CD has an impact on Dpp10-mediated modulation of Kv4-mediated A-type K^+ channels in aortic tissue.

In order to test whether protein expression levels of Dpp10, Car3, Cidec and Cyp2E1 are differentially expressed by HFD and HFD/CD Western Blot analysis was performed. Detection of Dpp10 protein was unsuccessful, because of unspecific binding of antibody against Dpp10. A Dpp10 blocking peptide could not resolve the problem (data not shown).

Car3 was detected at 28 kDa. Its expression is downregulated by HFD and HFD/CD which matches to the results obtained with qRT-PCR. This indicates that both diets impair mRNA synthesis leading to decreased expression of the protein. Another explanation would be that mRNA is posttranscriptional modified. It could be that one part of the mRNA is degraded and only a small part is transferred to the cytosol for protein translation. This would explain why protein expression of Car3 is generally lower in all three treatment groups compared to qRT-PCR data.

Cidec has a molecular weight of 27 kDa. Cidec mRNA is downregulated by HFD and HFD/CD whereas protein expression is downregulated by CD and upregulated by HFD and HFD/CD. This indicates that CD stabilizes mRNA of Cidec but translation of Cidec is repressed possibly by specific microRNAs (miRNAs) or Cidec is degraded posttranslational. The decrease in Cidec mRNA levels by HFD and HFD/CD could be due to a negative feedback inhibition leading to a degradation of Cidec mRNA initiated when Cidec protein is translated and stabilized.

Interestingly, full-length Cyp2E1 at 51 kDa could not be detected in aortic protein lysates, but 2 other bands could be detected at 39 and 45 kDa. Quantification of these two bands showed that expression of Cyp2E1 at 39 kDa is downregulated by HFD and HFD/CD and expression of Cyp2E1 at 45 kDa showed a tendency to be downregulated by both diets. This indicates the generation of at least 3 different mRNA transcripts. There are several different possibilities why Cyp2E1 full-length is not detected by Western Blot in aortic tissue: (i) *Cyp2E1* gene is not formed after splicing or (ii) it is formed but degraded or (iii) not translated into protein. The other two mRNA transcripts

are translated and expressed but different posttranscriptional and posttranslational regulations might play a role. From the qRT-PCR results, it is impossible to figure out from which mRNA transcript, mRNA expression levels are detected. In liver, Cyp2E1 expression is regulated posttranscriptional by stabilization of mRNA and posttranslational by protecting against protein degradation (Song *et al.*, 1987; Roberts *et al.*, 1995; Lieber, 1997). Mohri *et al*. showed that human Cyp2E1 is regulated by the miRNA miR-378 which needs to bind to the 3`UTR leading to translational repression thereby decreasing Cyp2E1 protein expression (Mohri *et al.*, 2010).

Interestingly, mRNA expression of *Car3*, *Cidec* and *Cyp2E1* were downregulated by HFD and HFD/CD whereas mRNA expression of *Dpp10* was upregulated by both diets which could also suggest that transcription of *Car3*, *Cidec* and *Cyp2E1* is decreased and transcription of *Dpp10* is increased by HFD and HFD/CD. Transcriptional regulation controls whether and how much mRNA is synthesized. Epigenetic processes can cause alterations in gene expression without altering DNA sequence. Such processes include DNA methylation or histone deacetylation which can lead to a decrease in transcription and gene expression (Gibney & Nolan, 2010). Diet-induced and genetically caused obesity in mice was reported to affect gene expression by epigenetic regulation (Takahashi *et al.*, 2005; Stoger, 2008). Additionally, activation of repressors binding to silencer regions in the promoter region of a gene does not allow RNA-polymerase to bind to the promoter thereby impairing transcriptional process (Lodish *et al.*, 2007). Certain transcription factors, called general transcription factors, are needed to start transcription because they help RNA polymerase to bind to the promoter (Lodish *et al.*, 2007). Therefore, a disruption in this transcription initiation complex can effect expression of a gene. It may be possible that HFD and HFD/CD affects the transcription machinery for *Car3*, *Cidec* and *Cyp2E1* to reduce their expression while it activates transcription for *Dpp10*.

Taken together, in aortic tissue high dietary fat intake leads in at least four genes to gene and protein expression changes that sustain after high-dietary fat is reduced.

4.4 Vascular Cyp2E1 splice variants

Alternative splicing of eukaryotic genes is important since it increases the variety of transcripts leading to various protein isoforms (Tazi *et al.*, 2009). Approximately 70-90 % of human genes are alternatively spliced (Johnson *et al.*, 2003; Wang *et al.*, 2008). Splicing is the process in which introns are removed from the transcribed pre-mRNA which can result in joining of exons in multiple ways. Products are alternatively spliced mRNAs leading consequently to different protein isoforms (Black, 2003). Alternative splicing events can be various: (I) exons can be included or excluded, known as cassette exon or exon skipping, (II) exclusion of exon mutually means that one exon of two will

be retained in the mRNA, but not both at the same time resulting in two alternatively spliced mRNAs (III) alternative 5` (a) and 3` (b) splice sites occur which may lead to a shorter or longer exon (IV) an alternative promoter (a), poly(A) region (b) and the removal of the stop codon (c) can have an impact on transcription and translation, (V) part of an intron or the whole intron can be retained in the generated mRNA (Black, 2003). The splicing process is very complex and includes multiple steps (Black, 2003). A defect in the splicing process in form of mutations can lead to diseases (Faustino & Cooper, 2003).

In this study, statistical analysis using Tukey`s post hoc test revealed gene that 4 exons (4, 7, 8, 9) of *Cyp2E1* are downregulated by HFD compared to CD, and 8 exons (2-9) are downregulated by HFD/CD compared to CD (see **Table 3-3**) Based on this analysis, an exon specific PCR was performed to test whether differentially expressed exons are due to the existence of truncated exons. Results showed that exons 1-8 are detectable in all dietary treatment groups, but their expressions are downregulated by HFD and HFD/CD in aortic tissue but not in liver as verified with qRT-PCR (data not shown). This is not in line with the Tukey`s post hoc data. Additionally, it indicates that differential exon expression is not necessarily related to truncation of an exon. Furthermore, data suggest that HFD and HFD/CD have an impact on expression of exons which is independent of their amplification. This means that the amount of cDNA amplified representing the amount of mRNA produced is independent of its expression. Interestingly, only exon 9 including the stop codon is amplified in all groups whereas 81 bp downstream of the stop codon in the 3`UTR is amplified in the CD group but amplification rate diminishes with HFD and HFD/CD. Furthermore, additional 92 bp in the 3`UTR are not amplified at all in any of the groups. This implicates that the end part of the 3`UTR is truncated in aortic tissue of all dietary treatment groups and suggests a spliced isoform independently of HFD and HFD/CD. 3 `UTR plays a role in mRNA stability, its localization in the cytoplasm and is involved in the regulation of translation (Mignone *et al.*, 2002; Mazumder *et al.*, 2003). It may suggest that mRNA of full-length Cyp2E1 is degraded or not functional because of partial truncated 3`UTR therefore full-length *Cyp2E1* is not translated. Important to note is that even though exons 1-8 are detectable it does not mean that splice variants do not exist. In liver, two truncated isoforms of Cyp2E1 were identified (Zhuge & Cederbaum, 2006a; b). Interestingly, in both truncated versions 229 bp of the 3`UTR from exon 9 are missing. This is partly in line with our data. In addition, variant 1 lacks exons 4-6 corresponding to 1012 bp and 37 kDa (Zhuge & Cederbaum, 2006a) while variant 2 lacks exons 1-6 and 35 bp from exon 7 corresponding to 484 bp and 17 kDa (Zhuge & Cederbaum, 2006b). In the present study, exon skipping analysis using a primer pair detecting exon 4-7 identified an isoform of Cyp2E1 in which exons 4-6 were skipped in aortic tissue. This would suggest that aortic Cyp2E1 isoform lacks exons 4-6 as shown for Cyp2E1 variant 1 in liver. Lack of exons 4-6 would account for an isoform length of ~1280 bp (full-length *Cyp2E1* nucleotide sequence: 1759 bp subtracted by 479 bp (exon 4-6) and a molecular

weight of ~47 kDa. This isoform was only detected in the CD group but not in the HFD and HFD/CD group. This might be due to decreased Cyp2E1 mRNA expression levels in the HFD and HFD/CD group (verified with qRT-PCR). Western Blot analysis detected two protein isoforms at 39 and 45 kDa in aorta of the HFD and HFD/CD group. Overall it is difficult to assess whether the two identified Cyp2E1 protein bands at 39 and 45 kDa can be correlated with the results of the exon-specific and exon skipping PCR analysis but it is possible that aortic Cyp2E1 splice variant lacking exons 4-6 corresponds to the Western Blot band at 45 kDa. To test this, identified transcript should have been cloned separately into an expression vector to further be transfected into a cell line to overexpress this isoform. Overexpressed Cyp2E1 isoform could be detected with Western Blots and be compared to the bands obtained in aortic tissue.

Taken together, the results indicate that two different *Cyp2E1* mRNA transcripts could be identified. Additionally, two new Cyp2E1 protein isoforms in aortic tissue are detected in all dietary treatment groups with reduced expression induced by HFD and HFD/CD. Full-length Cyp2E1 mRNA transcript is detectable by PCR but probably not translated into protein.

4.5 Effect of DETC and role of Cyp2E1 in vascular reactivity

Cyp2E1 acts as a ω- hydroxylase catalyzing the formation of 18 (*R*)-, 19 (*R*)-and 19(*S*)-HETEs from AA in the vasculature (Laethem *et al.*, 1993). Zhang *et al*. showed that inhibition of Cyp2E1 by DETC decreases levels of 18- and 19-HETE in interlobar arteries of SHR. These researchers also showed that a decrease in 18-and 19- HETE enhances Cyp4A-derived 20-HETE-mediated contractions and that 20-HETE itself enhances phenylephrine-mediated contractions (Zhang *et al.*, 2005) indicating that 18- and 19-HETE act as vasodilators. These findings are further strengthened by a recent publication demonstrating that streptozotocin-induced diabetes in rats increases the expression of Cyp2E1 and 18- and 19-HETE levels in aorta which decreased 20-HETE levels and phenylephrine-mediated contractions. These data indicate that up regulation of Cyp2E1 increases 18-and 19-HETE formation which decrease phenylephrine-mediated contractions by inhibition of 20-HETE. (Schafer *et al.*, 2010) Additionally, Cyp2E1 can generate ROS such as O_2^- and H_2O_2 (Lu & Cederbaum, 2008) during the "decoupling reaction" (Loida & Sligar, 1993). It is also important to note, that DETC is not only inhibiting Cyp2E1 but also is reported to inhibit SOD (Ghanam *et al.*, 1998; Karasu, 2000). SOD catalyzes O_2^- to H_2O_2. H_2O_2 is known to be a vasodilator. It can be further converted by catalase to H_2O and O_2 or to •OH by the Fenton (Dikalova *et al.*, 2001) or Haber-Weiss reaction (Ingelman-Sundberg & Johansson, 1984; Taniyama & Griendling, 2003; Kyaw *et al.*, 2004). Additionally, DETC is able to scavenge NO in the presence of exogenous and endogenous iron in cells and tissues which leads to an

impaired vasorelaxant response due to NO-releasing agonists such as acetylcholine (Vedernikov *et al.*, 1992).

In this present study, vascular function experiments using Cyp2E1$^{-/-}$ mice showed that DETC is not a specific Cyp2E1 inhibitor for aortic Cyp2E1, but rather inhibits SOD. This suggests that the results obtained with DETC should be interpreted with caution.

4.5.1 Effect of DETC in vascular reactivity of mice fed with diets of different fat content

Inhibition with DETC in aorta of the dietary treatment groups increased phenylephrine-induced contractions in the CD group and decreased contractions in the HFD and HFD/CD group. It can be speculated that in the CD group O_2^- is generated and immediately converted into H_2O_2 which possibly dilates phenylephrine-mediated contractions. Increased contractions to phenylephrine in the HFD and HFD/CD group might be due to decreased O_2^- production and conversion to H_2O_2.

Contrariwise, 5-hydroxytryptamine-induced contractions upon inhibition with DETC were decreased by CD and HFD/CD and were unaffected by HFD. It could be that O_2^- is converted into H_2O_2 and further into •OH. •OH has vasoconstrictor properties in aorta of rats (Li *et al.*, 2004). It is possible that increased contractile responses to 5-hydroxytryptamine in the CD and HFD/CD group involve •OH.

Endothelium-dependent contractions were reduced in aorta by inhibition with DETC in the CD and HFD group but not in the HFD/CD group. It is known that O_2^- is involved in endothelium-dependent contractions and that those contractions can be decreased by DETC (Vanhoutte, 2002) The results might implicate that O_2^- is involved in endothelium-dependent contractions in CD and HFD group and that probably high amounts of O_2^- are present in the HFD group.

In this study, changes in vascular reactivity due to dietary treatments are probably regulated by involvement of ROS.

4.5.2 Vascular reactivity in wild type and Cyp2E1-deficient mice

Results of vascular function experiments obtained with WT and Cyp2E1$^{-/-}$ mice showed that phenylephrine-induced contractions are uneffected. This suggests that Cyp2E1 plays no role in phenylephrine-mediated contractions in the analyzed experimental setting. Schäfer *et al.* showed that in aorta of diabetic rats Cyp2E1 expression is upregulated leading to increased levels of 18-and 19-HETE decreasing phenylephrine-mediated contractions by inhibition of 20-HETE. (Schafer *et al.*, 2010) It is possible that Cyp2E1 is only important in regulation of phenylephrine-induced contraction in diseased condition. Interestingly, inhibition with DETC increased contractions to phenylephrine in WT and Cyp2E1$^{-/-}$ mice which suggests that O_2^- levels are similar in both groups. It could be speculated that possibly O_2^- is converted to

similar levels of H_2O_2 and that phenylephrine-mediated contractions are controlled by dilatory properties of H_2O_2 in both groups.

Contractile responses to 5-hydroxytryptamine were decreased in Cyp2E1$^{-/-}$ mice. This indicates that Cyp2E1 has an influence on 5-hydroxytryptamine contractions but probably not by the involvement of dilatory metabolites. Inhibition of 5-hydroxytryptamine-induced contractions by DETC decreased only slightly, but significant in aorta of WT mice, whereas inhibition in the Cyp2E1$^{-/-}$ mice had no effect. This might explain that 5-hydroxytryptamine-mediated contractions are induced by generation of O_2^- through Cyp2E1 which is first converted into H_2O_2 and finally into •OH. •OH may contribute to an increased response to 5-hydroxytryptamine in WT mice.

Interestingly, acetylcholine-dependent relaxation was impaired in Cyp2E1$^{-/-}$ mice compared to WT mice indicating that Cyp2E1 contributes to acetylcholine-induced relaxation. A possible explanation would be that acetylcholine not only stimulates the release of NO but also activates Cyp2E1 to release 18-and 19-HETE which potentiate NO-induced endothelium-dependent relaxation. Interestingly, inhibition with DETC impaired acetylcholine-induced relaxation in WT mice (Omar *et al.*, 1991; Vedernikov *et al.*, 1992; Karasu, 2000) but no effect was observed in Cyp2E1$^{-/-}$ mice, suggesting that DETC either scavenges NO or inhibits SOD thereby increasing O_2^- levels which can also scavenge NO leading to decreased relaxant responses in WT-mice. Since L-NAME inhibits acetylcholine-induced relaxation in WT and Cyp2E1$^{-/-}$ mice (data not shown), it indicates that relaxant responses are NO-dependent in the presence and absence of Cyp2E1.

Endothelium-dependent contractions were enhanced in Cyp2E1$^{-/-}$ mice and were completely blocked by non-specific COX-inhibitor meclofenamate. These findings implicate that Cyp2E1 decreases endothelium-dependent contractions possibly by releasing 18-and 19-HETE which due to their dilatory properties decrease those contractions in WT mice. Furthermore, inhibition of endothelium-dependent contractions by DETC in WT mice suggests that O_2^- also contributes to those contractions. Moreover, it can be speculated that Cyp2E1 through 18-and 19-HETE decreases COX-activity in WT mice, because endothelium-dependent contractions in Cyp2E1$^{-/-}$ mice are COX-dependent. Interestingly, endothelium-dependent contractions in Cyp2E1$^{-/-}$ mice are not affected by DETC. It proposes that in the absence of Cyp2E1 endothelium-dependent contractions are not mediated by O_2^- but that these contractions are probably mediated by other factors which stimulate COX.

Taken together, Cyp2E1 modulates vascuar reactivity of aorta from mice possibly via ROS and NO availability.

4.6 Further directions

One of the most pivotal follow-up question of the present study is whether in Cyp2E1$^{-/-}$ mice vascular function is changed after HFD or HFD/CD compared to control mice. Intriguingly, data of an abstract presented by Armoni *et al*. in June 2011 (Armoni *et al.*, 2011) at the American Diabetes Association 71st Scientific Sessions showed that Cyp2E1$^{-/-}$ mice fed with a high-fat diet for 8 weeks were protected against obesity and had no impaired glucose tolerance compared to wild type mice fed a high fat diet. Furthermore, these researchers showed that Cyp2E1 induces insulin resistance via oxidative stress and inhibition of Glut4 expression.

To mechanistically understand the role of the oxidoreductase Cyp2E1 in the vasculature it will be important to determine in which conditions (i) its vasorelaxent products 18- or 19-HETEs are formed or, alternatively, (ii) uncoupling of the catalytic process generates ROS.

Another avenue of investigation could be to test whether identified Cyp2E1 splice variants are functional. To this end Cyp2E1 splice variants should be cloned to perform functional enzyme activity assays. It remains to be determined why Cyp2E1 splice variants and not full length version are expressed at protein level in the vasculature. An interlocking question could be to test the contribution of the two different isoforms (39 and 45 kDa) in the regulation of vascular reactivity.

Apart of *Cyp2E1* other genes (*Car3*, *Cidec*, and *Dpp10*) were shown to be differentially expressed by HFD and HFD/CD feeding. These molecules may also be of importance in regulation of vascular reactivity. It warrants further studies to delineate their impact on vascular reactivity.

4.7 Conclusion and clinical implications

In this dissertation, a vascular transcriptome analysis of mice fed with diets containing different fat contents was performed. Genes were identified with expressional changes that sustain after high-dietary fat content is reduced. One of the target genes was *Cyp2E1*, shown to play a role in regulating vascular reactivity. Possible mechanisms by which Cyp2E1 regulates vascular reactivity in aorta involve ROS and NO. Based on these data it could be speculated that a downregulation of gene and protein expression of aortic Cyp2E1 by HFD and HFD/CD implicates that a compensatory mechanism protects from diet-induced changes in the vasculature. An upregulation of Cyp2E1 in aortic tissue by HFD and HFD/CD would probably damage the vasculature heavily since it is known that increased expression of Cyp2E1 by

chronic ethanol consumption leads to generation of oxidative stress which is strongly associated with liver injury and subsequently development of alcoholic liver disease (Lieber, 1997). Therefore, the results would implicate that alterations in vascular reactivity due to HFD and HFD/CD are caused by other ROS producing enzymes than Cyp2E1. Furthermore, this interpretation would be strengthened by results of Armoni *et al.* showing that Cyp2E1$^{-/-}$ mice fed with a high-fat diet for 8 weeks were protected against obesity and had no impaired glucose tolerance compared to wild type mice fed a high fat diet (Armoni *et al.*, 2011). It can be speculated that Cyp2E1$^{-/-}$ mice fed a high-fat diet would be protected against diet-induced changes in vascular reactivity.

Obesity is usually associated with a decrease in antioxidant enzymes (Ozata *et al.*, 2002). Researches showed that impaired endothelium-dependent relaxation in aorta of rats due to high-fat diet feeding for 6 months could be preserved after high-fat diet feeding supplemented with antioxidants vitamin A and E (Sato *et al.*, 2002). However, clinical trials showed that longer treatment with antioxidant vitamins did not improve cardiovascular diseases (Forstermann, 2008). On the other hand, treatment with statins inhibits endothelial O_2^- formation and increases expression of eNOS (Forstermann, 2008). Anti-obesity drugs such as orlistat and sibutramine are successfully used in reduction of body weight in obese patients and in improvement of endothelial function (Sekuri *et al.*, 2003; Shechter *et al.*, 2006). Important to note is that side effects occur using those drugs. For example, sibutramine treatment increases blood pressure (McNeely & Goa, 1998). Interestingly, Ozcelik *et al.* reported that obese patients maintained on a hypocaloric diet combined with orlistat treatment and exercise training for 12 weeks reduced body weight but also levels of malonaldehyde, a marker for lipid peroxidation (Ozcelik *et al.*, 2005). It would be important to develop anti-obesity drugs that decrease oxidative stress and specifically target enzymes that produce oxidative stress in the vasculature. Another possible therapeutic approach to improve diet-induced changes in vascular reactivity due to increased ROS in obese patients and in those who were obese would be proteasome inhibition. It is known that proteasome inhibition increases expression of antioxidative enzymes and protects against oxidative stress (Yamamoto *et al.*, 2007). Recently, it was shown that proteasome inhibitor PS-341 (Bortezomib, Velcade®) is effective in the treatment of alcohol liver disease (Bardag-Gorce, 2011). In liver of rats fed with ethanol, antioxidative enzymes were downregulated. After treatment with proteosome inhibitor PS-341 expression of antioxidant enzymes increased. This suggest that PS-341 is a potential drug for treatment of alcoholic liver disease induced by oxidative stress. It would be important to test, whether PS-341 treatment in obese patients and those who were obese improves diet-induced changes in vascular reactivity.

An alternative interpretation of the data is as follows: Downregulation of gene and protein expression of aortic Cyp2E1 by HFD and HFD/CD is not due to a compensatory mechanism to protect the vasculature but rather responsible for diet-induced changes in vascular reactivity. This would implicate that aortic Cyp2E1 under physiological

condition has a protective role against diet-induced changes in the vasculature. Following this interpretation it would be important to increase expression of aortic Cyp2E1 in obese patients and in those who were obese. Drugs have to be developed to block diet-induced downregulation of Cyp2E1 by manipulation of the transcription machinery. This approach might be difficult to implement because Cyp2E1 is a multifunctional enzyme and its expression is differently regulated by diseases.

Taken together, these data could provide important information for the development of new strategies to treat or prevent obesity-associated pathophysiological changes in the vasculature in humans.

5 Appendix

5.1 Oligonucleotide primers used in qRT-PCR for amplification of mouse cDNA

Gene **NCBI mRNA Accession Number**	Oligonucleotide Forward Primer Oligonucleotide Reverse Primer	Product Size (bp)	Exon Covered by Primer Pair
Actb **NM_007393**	5‘-CGTGCGTGACATCAAAGAGA-3‘ 5‘-CCCAAGAAGGAAGGCTGGA-3‘	180	Exon 3
Car3 **NM_007606**	5‘-CACACTTTGACCCATCATGC-3‘ 5‘-AGCTCACAGTCATGGGCTCT-3‘	129	Exon 6
Cidec **NM_178373**	5‘-GACCTCCTGAACAAGGTCCA-3‘ 5‘-ATTGTGCCATCTTCCTCCAG-3‘	80	Exon 3-4
Cyp2E1 **NM_021282**	5‘-CTCTGAGATATGGGCTCCTGA-3‘ 5‘-CAACTGTACCCTTGGGGATG-3‘	244	Exon 7
Dgkg **NM_138650**	5‘-TGAGCACACACCTCTTCCTG-3‘ 5‘-CATCTGCTTTGGCAGCATTA-3‘	132	Exon 4-5
Dpp10 **NM_199021**	5‘-GAGGCCAAATGGTGACAGAT-3‘ 5‘-CTGGAATCCACTTCCTCTGC-3‘	107	Exon 20
Fkbp5 **NM_010220**	5‘-GCTGTGGTGGAAGGACATTT-3‘ 5‘-CATCTTCACCAGGGCTTTGT-3‘	104	Exon 6
Fmo3 **NM_008030**	5‘-GGGGGAAAAGTTCAAATGGT-3‘ 5‘-CCTGGGATCCTTGAGAAACA-3‘	130	Exon 8-9
Lepr **NM_001122899**	5‘-GGGAATGAGCAAGGTCAAAA-3‘ 5‘-TCAAGTCCCCTTTCATCCAG-3‘	139	Exon 3
Lyve1 **NM_053247**	5‘-CATCCCTCGGATTTTCTCAA-3‘ 5‘-TCGGATGAGTTGTGGCAATA-3‘	111	Exon 3
Nrg1 **NM_178591**	5‘-GAATACGAAACGACCCAGGA-3‘ 5‘-GAACTTGGGTTGCTGTCCAT-3‘	134	Exon 2-3
Pdk4 **NM_013743**	5‘-TGACTCAAAGACGGGAAACC-3‘ 5‘-ACTGGTCGCAGAGCATCTTT-3‘	110	Exon 5-6
P2rx1 **NM_008771**	5‘-ACTGGGAGTGTGACCTGGAC-3‘ 5‘-CAGGTTCTTCTCCCCGTACA-3‘	83	Exon 8

Table 5-1 Oligonucleotide primers used for validation of target genes using qRT-PCR
Gene symbols, NCBI mRNA accession numbers, nucleotide sequences of the gene specific oligonucleotide forward and reverse primers, product sizes of the gene amplicons in basepairs (bp), binding position of primer pairs at exons of specific genes are listed. Primer pairs of *Cidec*, *Dgkg*, *Fmo3*, *Nrg1*, and *Pdk4* bind between end and start of two exons.

5.2 Oligonucleotide primers used in *Cyp2E1*"exon-specific PCR"

Cyp2E1 **Exon Region**	Oligonucleotide Forward Primer Oligonucleotide Reverse Primer	Product Size (bp)
Exon 1	5'-CATCACCGTTGCCTTGCT-3' 5'-GACCCAGGTGCAGTGTGAA-3'	207
Exon 2	5'-TTTCAGCTGGATTTGAAGGA-3' 5'-CCTTCCATGTGGGTCCATTA-3'	235
Exon 3	5'-GGGACATTCCTGTGTGTTCCAG-3' 5'-GCACAGCCAATCAGAAAGGT-3'	223
Exon 4	5'-CTGGTGGAGGAGCTCAAAAA-3' 5'-CTTTTCTGTGGCTTCCAGGT-3'	244
Exon 5	5'-TGGAGCTCATGAGTTTGTTCA-3' 5'-ACATGGGTTCTTGGCTGTGT-3'	261
Exon 6	5'-GGGATGTGACTGACTGTCTCC-3' 5'-GGCCCAATAACCCTGTCAAT-3'	217
Exon 7	5'-CTCTGAGATATGGGCTCCTGA-3' 5'-CAACTGTACCCTTGGGGATG-3'	244
Exon 8	5'-CGTGTTCCGAGGATATGTCA-3' 5'-AGGCCTTCTCCAACACACAC-3'	198
Exon 9/3_Stop	5'-CCTGAGCATTTTCTGAATGAA-3' 5'-GGTCTCATGAACGAGGAATGA-3'	256
Exon 9/2_3`UTR	5'-CCTGAGCATTTTCTGAATGAA-3' 5'-GAGCAGTCGGGTGCTCTTAC-3'	337
Exon 9/1_3`UTR	5'-CCTGAGCATTTTCTGAATGAA-3' 5'-GCTGTCTTCAGACACTCCAGAA-3'	435

Table 5-2 *Cyp2E1* exon-spanning oligonucleotide primers for identification of *Cyp2E1* splice vaiants in aorta using PCR
Table contains *Cyp2E1* exon regions, nucleotide sequences of oligonucleotide forward and reverse primers and expected PCR product sizes in basepairs (bp). Oligonucleotide forward and reverse primers are designed as exon-spanning primers where each primer pair covers one exon. For analyzing exon 9 three different primer pairs were designed. The forward primer is the same in all three primer pairs. Exon 9/3_Stop reverse primer binds to the stop codon of *Cyp2E1*, Exon 9/2_UTR and Exon 9/1_UTR reverse primers bind at different positions in the 3`untranslated region of *Cyp2E1*.

5.3 Experimental set up: Investigation of aortic function in mice *ex vivo*

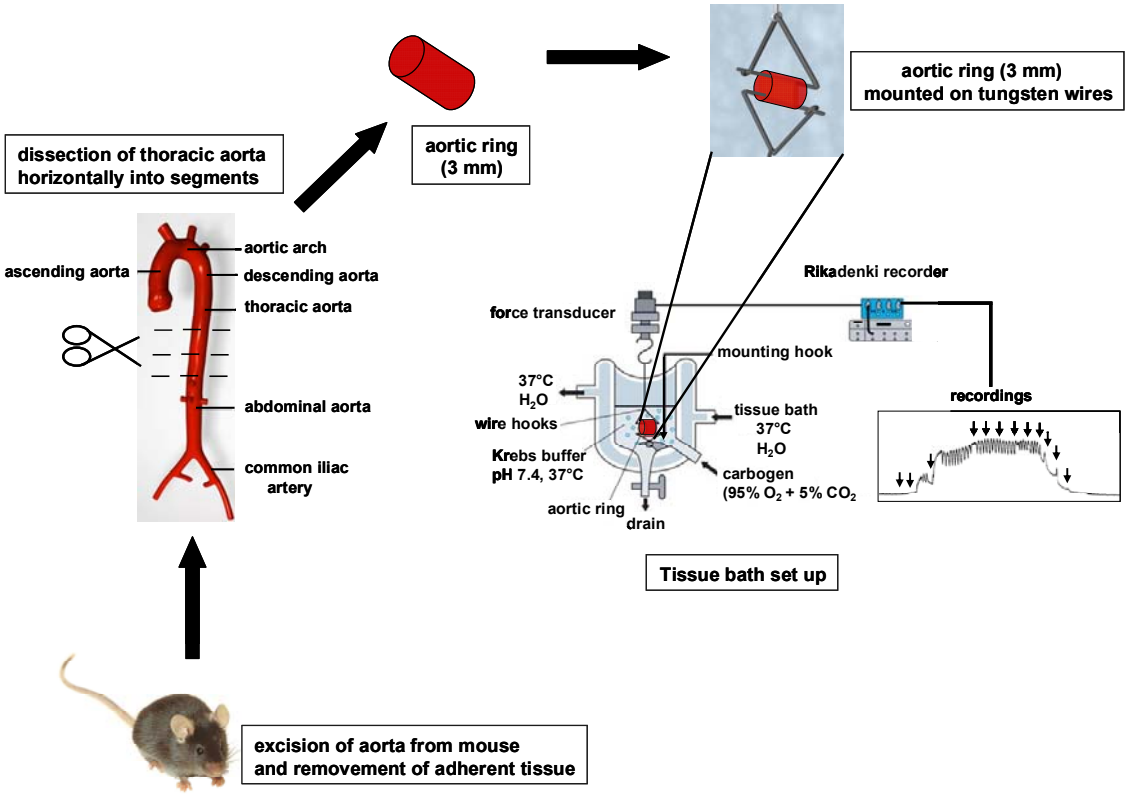

Figure 5-1 Course of vascular function experiments
First, the experiment begins with euthanasia of the mouse before aorta is excised and cleaned from attached adherent tissue. Then, thoracic aorta is dissected horizontally into aortic rings of 3 mm in size before mounted onto tungsten wires and transferred to tissue baths containing pre-warmed Krebs buffer. Rings are connected to force transducers which are connected to Rikadenki recorders to plot changes in vascular reactivity.

5.3.1 Representative recordings of vascular contraction and relaxation curves

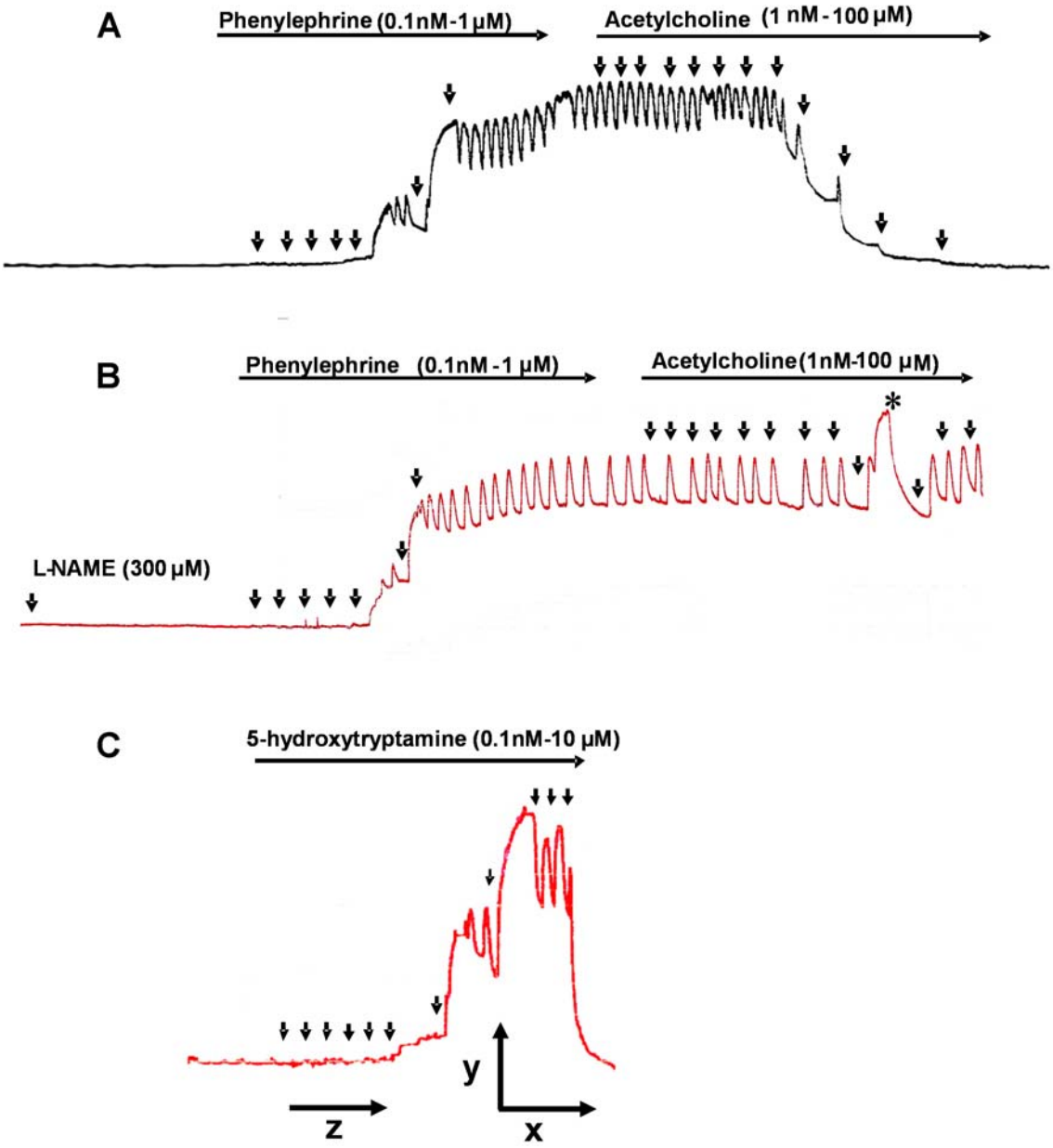

Figure 5-2 Representative recording for contractile and relaxant responses in thoracic aorta
(A) This graph represents a typical protocol used to investigate phenyleprine-mediated contractions and acetylcholine-induced relaxation **(B)**. Contractile responses to phenylephrine and acetylcholine-induced endothelium-dependent contraction indicated by a * **(C)**. Contractile response to 5-hydroxytryptamine is shown. z indicates the direction of the protocol, x corresponds to 10 min, y to 0.5 g. Vertical arrows indicate cumulative concentrations of phenylephrine and acetylcholine **(A)**, **(B)** , and 5-hydroxytryptamine **(C)**.

5.4 Coverage of exons by probe sets

Gene	Probe set Exon	Total Number of Exons
Car3 →	1 2 3 4 5 6 7 8 9 10 1 2 3 4 5 6 7 7 7 7	7
Cidec ←	1 2 3 4 5 6 7 6 6 5 4 3 2 1	6
Cyp2E1 →	1 2 3 4 5 6 7 8 9 10 11 12 1 1 2 3 4 5 6 7 7 8 9 9	9
Dgkg ←	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 25 25 23 23 22 21 20 19 18 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 2 1	25
Dpp10 ←	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 26 26 26 25 24 23 22 21 20 19 18 16 14 13 12 11 10 9 8 7 6 5 4 3 2 1	26
Fkbp5 ←	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 11 11 11 11 10 9 8 7 6 5 4 3 2 1 1 1 1 1	11
Fmo3 ←	1 2 3 4 5 6 7 8 9 10 9 9 9 8 7 6 5 4 3 2	9
Lepr →	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 1 1 1 2 3 4 5 6 7 8 9 10 11 12 13 15 15 16 18 19 20 20 22 22	22
Lyve1 ←	1 2 3 4 5 6 7 8 9 10 6 6 6 5 4 3 3 2 2 1	6
Nrg1 ←	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 9 9 8 8 7 6 5 3 3 2 1 1 1 1 1 1 1 1	9
Pdk4 ←	1 2 3 4 5 6 7 8 9 10 11 12 13 11 11 10 9 8 7 6 5 4 3 2 1 1	11
P2rx1 →	1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6 7 8 9 11 12 12	12

Table 5-3 Coverage of exons of a gene by probe sets
Represented are number of probe sets and total number of exons per gene. One exon can be covered by more than one probe set. Arrows indicate 5`to 3`direction of the gene.

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